

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
4 October 2001 (04.10.2001)

PCT

(10) International Publication Number  
WO 01/73093 A2(51) International Patent Classification<sup>7</sup>: C12N 15/86,  
15/63, 5/10, A61K 48/00, A61P 35/00

(74) Agents: GLAISTER, Debra, J. et al.; Morrison &amp; Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

(21) International Application Number: PCT/US01/09036

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(22) International Filing Date: 21 March 2001 (21.03.2001)

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(25) Filing Language: English

## Published:

(26) Publication Language: English

— without international search report and to be republished upon receipt of that report

(30) Priority Data:  
60/192,156 24 March 2000 (24.03.2000) US

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(71) Applicant (for all designated States except US): CALYDON, INC. [US/US]; 1324 Chesapeake Terrace, Sunnyvale, CA 94089 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): YU, De-Chao [CN/US]; 352 Dolphin Isle, Foster City, CA 94404 (US). LI, Yuanhao [CA/US]; 568 Irvin Court, Palo Alto, CA 94306 (US). HENDERSON, Daniel, R. [US/US]; 955 Matadero Avenue, Palo Alto, CA 94306 (US).



A2

WO 01/73093

(54) Title: CELL-SPECIFIC ADENOVIRUS VECTORS COMPRISING AN INTERNAL RIBOSOME ENTRY SITE

(57) Abstract: Disclosed herein are replication-competent adenovirus vectors comprising co-transcribed first and second genes under transcriptional control of a heterologous, target cell-specific transcriptional regulatory element (TRE), wherein the second gene is under translational control of an internal ribosome entry site. Methods for the preparation and use of such vectors are also provided. The vectors provide target cell-specific virus replication in applications such as cancer therapy and gene therapy.

## CELL-SPECIFIC ADENOVIRUS VECTORS COMPRISING AN INTERNAL RIBOSOME ENTRY SITE

### TECHNICAL FIELD

5 This invention relates to new replication competent adenovirus vectors comprising an internal ribosome entry site which replicate preferentially in target cells. The present invention also relates to cell transduction using adenovirus vectors comprising an internal ribosome entry site.

### BACKGROUND

10 Diseases involving altered cell proliferation, particularly hyperproliferation, constitute an important health problem. For example, despite numerous advances in medical research, cancer remains the second leading cause of death in the United States. In the industrialized nations, roughly one in five persons will die of cancer. Traditional modes 15 of clinical care, such as surgical resection, radiotherapy and chemotherapy, have a significant failure rate, especially for solid tumors. Neoplasia resulting in benign tumors can usually be completely cured by surgical removal of the tumor mass. If a tumor becomes malignant, as manifested by invasion of surrounding tissue, it becomes much more difficult to eradicate. Once a malignant tumor metastasizes, it is much less likely to 20 be eradicated.

Excluding basal cell carcinoma, there are over one million new cases of cancer per year in the United States alone, and cancer accounts for over one half million deaths per year in this country. In the world as a whole, the five most common cancers are those of lung, stomach, breast, colon/rectum, and uterine cervix, and the total number of new cases 25 per year is over 6 million.

In the United States, transitional cell carcinoma (TCC) accounts for 90 to 95 percent of all tumors of the bladder. Squamous cell carcinoma (SCC) represents 5 to 10 percent, and adenocarcinoma approximately 1 to 2 percent. Squamous cell and adenomatous elements are often found in association with transitional cell tumors, especially with high 30 grade tumors. Bladder cancer is generally divided into superficial and invasive disease. A critical factor is the distinction between those tumors that are confined to the mucosa and those that have penetrated the basement membrane and extended into the lamina propria.

The term "superficial bladder tumor" is generally used to represent a tumor that has not invaded the muscularis. Invasive tumors are described as those that have invaded the muscularis propria, the perivesical fibroadipose tissue, or adjacent structures. Carcinoma in situ (CIS) is a high grade and aggressive manifestation of TCC of the bladder that has a 5 highly variable course.

A number of urothelial cell-specific proteins have been described, among which are the uroplakins. Uroplakins (UP), including UPIa and UPIb (27 and 28 kDa, respectively), UPII (15 kDa), and UPIII (47 kDa), are members of a group of integral membrane proteins that are major proteins of urothelial plaques. These plaques cover a large portion of the 10 apical surface of mammalian urothelium and may play a role as a permeability barrier and/or as a physical stabilizer of the urothelial apical surface. Wu et al. (1994) *J. Biol. Chem.* 269:13716-13724. UPs are bladder-specific proteins, and are expressed on a significant proportion of urothelial-derived tumors, including about 88% of transitional cell carcinomas. Moll et al. (1995) *Am. J. Pathol.* 147:1383-1397; and Wu et al. (1998) *Cancer Res.* 58:1291-1297. The control of the expression of the human UPII has been studied, and a 3.6-kb region upstream of the mouse UPII gene has been identified which can confer 15 urothelial-specific transcription on heterologous genes (Lin et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:679-683). See also, U.S. Patent Nos. 5,824,543 and 6,001,646.

Melanoma, a malignant neoplasm derived from melanocytes of the skin and other 20 sites, has been increasing in incidence worldwide. The American Joint Committee on Cancer recognizes five different forms of extraocular melanoma occurring in humans: lentigo maligna melanoma; radial spreading; nodular; acral lentiginous; and unclassified. Known melanoma-associated antigens can be classified into three main groups: tumor-associated testis-specific antigens MAGE, BAGE, GAGE, and PRAME; melanocyte 25 differentiation antigens tyrosinase, Melan-A/MART-1 (for Melanoma Antigen Recognized by T cells), gp100, tyrosinase related protein-1(TRP-1), tyrosinase related protein-2 (TRP-2); and mutated or aberrantly expressed antigens MUM-1, cyclin-dependent kinase 4 (CDK4), beta-catenin, gp100-in4, p15, and N-acetylglucosaminyltransferase V. See, for example, Kirkin et al. (1998) *Exp. Clin. Immunogenet.* 15:19-32. Tyrosinase, TRP-1, and 30 TRP-2 are enzymes involved in melanin biosynthesis and are specifically expressed in melanocytes. Antigenic epitopes of MART-1 have been studied extensively, with the aim of developing a melanoma vaccine. An immunodominant epitope, MART-1(27-35) has been reported to be recognized by a majority of CD8+ cytotoxic T cell clones generated to

MART-1. These MART-1(27-35)-specific CTLs specifically lyse autologous tumor cell lines expressing the epitope. Faure and Kourilsky (1998) *Crit. Rev. Immunol.* 18:77-86. However, others have reported that presence of such CTLs is not accompanied by a significant clinical response. Rivoltini et al. (1998) *Crit. Rev. Immunol.* 18:55-63.

5 A major, indeed the overwhelming, obstacle to cancer therapy is the problem of selectivity; that is, the ability to inhibit the multiplication of tumor cells without affecting the functions of normal cells. For example, in traditional chemotherapy of prostate cancer, the therapeutic ratio, (i.e., the ratio of tumor cell killing to normal cell killing) is only 1.5:1. Thus, more effective treatment methods and pharmaceutical compositions for therapy and 10 prophylaxis of neoplasia are needed.

Accordingly, the development of more specific, targeted forms of cancer therapy, especially for cancers that are difficult to treat successfully, is of particular interest. In contrast to conventional cancer therapies, which result in relatively non-specific and often serious toxicity, more specific treatment modalities, which inhibit or kill malignant cells 15 selectively while leaving healthy cells intact, are required.

Gene therapy, whereby a gene of interest is introduced into a malignant cell, has been attempted as an approach to treatment of many cancers. See, for example, Boulikas (1997) *Anticancer Res.* 17:1471-1505, for a description of gene therapy for prostate cancer. A gene of interest can encode a protein which is converted into a toxic substance upon 20 treatment with another compound, or it can encode an enzyme that converts a prodrug to a drug. For example, introduction of the herpes simplex virus gene encoding thymidine kinase (HSV-tk) renders cells conditionally sensitive to ganciclovir. Zjilstra et al. (1989) *Nature* 342: 435; Mansour et al. (1988) *Nature* 336: 348; Johnson et al. (1989) *Science* 245: 1234; Adair et al. (1989) *Proc. Natl. Acad. Sci. USA* 86: 4574; Capecchi (1989) *Science* 244: 1288. Alternatively, a gene of interest can encode a compound that is directly 25 toxic, such as, for example, diphtheria toxin. To render these treatments specific to cancer cells, the gene of interest is placed under control of a transcriptional regulatory element (TRE) that is specifically (i.e., preferentially) active in the cancer cells. Cell- or tissue-specific expression can be achieved by using a TRE with cell-specific enhancers and/or 30 promoters. See generally Huber et al. (1995) *Adv. Drug Delivery Reviews* 17:279-292.

A number of viral vectors and non-viral delivery systems (e.g., liposomes), have been developed for gene transfer. Of the viruses proposed for gene transfer, adenoviruses are among the most easily produced and purified. Adenovirus also has the advantage of a

high efficiency of transduction (*i.e.*, introduction of the gene of interest into the target cell) and does not require cell proliferation for efficient transduction. In addition, adenovirus can infect a wide variety of cells *in vitro* and *in vivo*. For general background references regarding adenovirus and development of adenoviral vector systems, see Graham *et al.*

5 (1973) *Virology* 52:456-467; Takiff *et al.* (1981) *Lancet* 11:832-834; Berkner *et al.* (1983) *Nucleic Acid Research* 11: 6003-6020; Graham (1984) *EMBO J* 3:2917-2922; Bett *et al.* (1993) *J. Virology* 67:5911-5921; and Bett *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806.

Adenoviruses generally undergo a lytic replication cycle following infection of a host cell. In addition to lysing the infected cell, the replicative process of adenovirus blocks the transport and translation host cell mRNA, thus inhibiting cellular protein synthesis. For a review of adenoviruses and adenovirus replication, see Shenk, T. and Horwitz, M.S., *Virology*, third edition, Fields, B.N. *et al.*, eds., Raven Press Limited, New York (1996), Chapters 67 and 68, respectively.

10 When used for gene transfer, adenovirus vectors are often designed to be replication-defective and are thus deliberately engineered to fail to replicate in the target cell. In these vectors, the early adenovirus gene products E1A and/or E1B are often deleted, and the gene to be transduced is commonly inserted into the E1A and/or E1B region of the deleted virus genome. Bett *et al.* (1994) *supra*. Such vectors are propagated in packaging cell lines such as the 293 line, which provides E1A and E1B functions in *trans*. Graham *et al.* (1987) *J. Gen. Virol* 36:59-72; Graham (1977) *J. Gen. Virol.* 68:937-940. The use of replication-defective adenovirus vectors as vehicles for efficient transduction of genes has been described by, *inter alia*, Stratford-Perricaudet (1990) *Human Gene Therapy* 1:241-256; Rosenfeld (1991) *Science* 252:431-434; Wang *et al.* (1991) *Adv. Exp. Med. Biol.* 309:61-66; Jaffe *et al.* (1992) *Nature Gen.* 1:372-378; Quantin *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584; Rosenfeld *et al.* (1992) *Cell* 68:143-155; Stratford-Perricaudet *et al.* (1992) *J. Clin. Invest.* 90:626-630; Le Gal Le Salle *et al.* (1993) *Science* 259:988-990; Mastrangeli *et al.* (1993) *J. Clin. Invest.* 91:225-234; Ragot *et al.* (1993) *Nature* 361:647-650; Hayashi *et al.* (1994) *J. Biol. Chem.* 269:23872-23875; and Bett *et al.* (1994) *supra*.

In the treatment of cancer by replication-defective adenoviruses, the host immune response limits the duration of repeat doses at two levels. First, the capsid proteins of the adenovirus delivery vehicle itself are immunogenic. Second, viral late genes are frequently

expressed in transduced cells, eliciting cellular immunity. Thus, the ability to repeatedly administer cytokines, tumor suppressor genes, ribozymes, suicide genes, or genes which convert a prodrug to an active drug has been limited by the immunogenicity of both the gene transfer vehicle and the viral gene products of the transfer vehicle, coupled with the 5 transient nature of gene expression. Despite these limitations, development of adenoviral vectors for gene therapy has focused almost exclusively on the use of the virus as a vehicle for introducing a gene of interest, not as an effector in itself. In fact, replication of adenovirus vectors has been viewed as an undesirable result, largely due to the host immune response.

10 More recently, however, the use of adenovirus vectors as effectors has been described. International Patent Application Nos. PCT/US98/04080, PCT/US98/04084, PCT/US98/04133, PCT/US98/04132, PCT/US98/16312, PCT/US95/00845, PCT/US96/10838, PCT/EP98/07380 and U.S. Pat. No. 5,998,205. Adenovirus E1A and E1B genes are disclosed in Rao *et al.* (1992, *Proc. Natl. Acad. Sci. USA* vol. 89: 7742-15 7746).

20 Replication-competent adenovirus vectors, which take advantage of the cytotoxic effects associated with adenovirus replication, have recently been described as agents for effecting selective cell growth inhibition. In such systems, a cell-specific transcriptional regulatory element (TRE) is used to control the expression of a gene essential for viral replication, thus limiting viral replication to cells in which the TRE is functional. *See*, for example International Patent Application No. PCT/EP99/07380, Henderson *et al.*, U.S. Patent No. 5,698,443; Hallenbeck *et al.*, PCT/US95/15455 and U.S. Patent No. 5,998,205; Rodriguez *et al.* (1997) *Cancer Res.* 57:2559-2563.

25 PCT publication PCT/US98/04080 discloses replication-competent, target cell-specific adenovirus vectors comprising heterologous TREs, such as those regulating expression of prostate-specific antigen (PSA), probasin (PB),  $\alpha$ -fetoprotein (AFP), kallikrein (*hKLK2*), mucin (*MUC1*) and carcinoembryonic antigen (CEA). PCT/US98/04084 discloses replication-competent adenovirus vectors comprising an  $\alpha$ -fetoprotein (AFP) TRE that replicate specifically in cells expressing AFP, such as 30 hepatoma cells.

Internal ribosome entry sites (IRES) are sequences which initiate translation from an internal initiation codon (usually AUG) within a bi-or multi- cistronic RNA transcript

continuing multiple protein coding regions. IRES have been characterized in encephalomyocarditis virus and related picornaviruses. *See, for example, Jackson et al. (1995) RNA 1: 985-1000 and Herman (1989) Trends in Biochemical Sciences 14(6): 219-222.* IRES sequences are also detected in mRNAs from other viruses such as cardiovirus, 5 rhinovirus, aphthovirus, hepatitis C virus (HCV), Friend murine leukemia virus (FrMLV) and Moloney murine leukemia virus (MoMLV). The presence of IRES in cellular RNAs has also been described. Examples of cellular mRNAs containing IRES include those encoding immunoglobulin heavy-chain binding protein (BiP), vascular endothelial growth factor (VEGF), fibroblast growth factor 2, insulin-like growth factor, translational initiation 10 factor eIF4G, and the yeast transcription factors TFIID and HAP4. *See, for example, ; Macejak et al. (1991) Nature 353:90-94; Oh et al. (1992) Genes Dev. 6:1643-1653; Vagner et al. (1995) Mol. Cell. Biol. 15:35-44; He et al. (1996) Proc. Natl. Acad. Sci USA 93:7274-7278; He et al. (1996) Gene 175:121-125; Tomanin et al. (1997) Gene 193:129-140; Gambotto et al. (1999) Cancer Gene Therapy 6:45-53; Qiao et al. (1999) Cancer 15 Gene Therapy 6:373-379.* Expression vectors containing IRES elements have been described. *See, for example, International Patent Application No. PCT/US98/03699 and International Patent Application No. PCT/EP98/07380.*

Thus, there is a continuing need for improved replication-competent adenovirus vectors in which cell-specific replication can be further enhanced, while minimizing the 20 extent of replication in non-target (*i.e.*, non-cancerous cells).

The disclosure of all patents and publications cited herein are incorporated by reference in their entirety.

#### SUMMARY OF THE INVENTION

25 The present invention provides improved replication competent adenovirus vectors comprising co-transcribed first and second genes under transcriptional control of a heterologous, target cell-specific transcriptional regulatory element (TRE), wherein the second gene is under translational control of an internal ribosome entry site (IRES). In one embodiment, the first and second genes are co-transcribed as a single mRNA and the 30 second gene has a mutation in or deletion of its endogenous promoter. The present invention further provides host cells and methods using the adenovirus vectors.

In one aspect, the first and/or second genes are adenovirus genes and in another aspect, the first and/or second adenovirus genes are essential for viral replication. An

essential gene can be an early viral gene, including for example, E1A; E1B; E2; and/or E4, or a late viral gene. In another aspect an early gene is E3.

In one embodiment, the first gene is an adenovirus gene and the second gene is a therapeutic gene. In another embodiment, both genes are adenovirus genes. In an 5 additional embodiment, the first adenovirus gene is E1A, and the second adenovirus gene is E1B. Optionally, the endogenous promoter for one of the co-transcribed adenovirus gene essential for viral replication, such as for example, E1A, is deleted and/or mutated such that the gene is under sole transcriptional control of a target cell-specific TRE.

In another aspect, the present invention provides adenovirus vectors comprising an 10 adenovirus gene essential for viral replication under control of a target cell-specific TRE, wherein said adenovirus gene has a mutation of or deletion in its endogenous promoter. In one embodiment, the adenovirus gene is essential for viral replication. In another embodiment, the adenovirus gene is E1A wherein the E1A promoter is deleted and wherein the E1A gene is under transcriptional control of a heterologous cell-specific TRE. In 15 another embodiment, the adenovirus gene is E1B wherein the E1B promoter is deleted and wherein the E1B gene is under transcriptional control of a heterologous cell-specific TRE.

In another aspect, the present invention provides adenovirus vectors comprising E1B under control of a target cell-specific TRE, wherein said E1B has a deletion in or mutation of the 19-kDa region of E1B, that encodes a product shown to inhibit apoptosis.

20 In other embodiments, an enhancer element for the first and/or second adenovirus genes is inactivated. The present invention provides an adenovirus vector comprising E1A wherein an E1A enhancer is inactivated. In yet other embodiments, the present invention provides an adenovirus vector comprising E1A wherein the E1A promoter is inactivated and E1A enhancer I is inactivated. In further embodiments, the present invention provides 25 an adenovirus vector comprising a TRE which has its endogenous silencer element inactivated.

Any TRE which directs cell-specific expression can be used in the disclosed vectors. In one embodiment, TREs include, for example, TREs specific for prostate cancer cells, breast cancer cells, hepatoma cells, melanoma cells, bladder cells and/or colon cancer cells. In another embodiment, the TREs include, probasin (PB) TRE; prostate-specific 30 antigen (PSA) TRE; mucin (MUC1) TRE;  $\alpha$ -fetoprotein (AFP) TRE; hKLK2 TRE; tyrosinase TRE; human uroplakin II TRE (hUPII) and carcinoembryonic antigen (CEA)

TRE. In other embodiments, the target cell-specific TRE is a cell status-specific TRE. In yet other embodiments, the target cell-specific TRE is a tissue specific TRE.

In additional embodiments, the adenovirus vector comprises at least one additional co-transcribed gene under the control of the cell-specific TRE. In another embodiment, an 5 additional co-transcribed gene is under the translational control of an IRES.

In another aspect of the present invention, adenovirus vectors further comprise a transgene such as, for example, a cytotoxic gene. In one embodiment, the transgene is under the transcriptional control of the same TRE as the first gene and second genes and optionally under the translational control of an internal ribosome entry site. In another 10 embodiment, the transgene is under the transcriptional control of a different TRE that is functional in the same cell as the TRE regulating transcription of the first and second genes and optionally under the translational control of an IRES.

The present invention also provides compositions comprising the replication-competent adenovirus vectors described herein. In one embodiment, the compositions 15 further comprise a pharmaceutically acceptable excipient. The present invention also provides kits comprising the replication-competent adenovirus vectors described herein.

Host cells comprising the disclosed adenovirus vectors are also provided. Host cells include those used for propagation of a vector and those into which a vector is introduced for therapeutic purposes.

In another aspect, methods are provided for propagating replication-competent adenovirus vectors of the present invention specific for mammalian cells which permit the 20 function of a target cell-specific TRE, said method comprising combining an adenovirus vector(s) described herein with mammalian cells that permit the function of a target cell-specific TRE, such that the adenovirus vector(s) enters the cell, whereby said adenovirus is propagated.

In another aspect, methods are provided for conferring selective cytotoxicity in target cells, comprising contacting the cells with an adenovirus vector(s) described herein, whereby the vector enters the cell.

The invention further provides methods of suppressing tumor cell growth, more 30 particularly a target tumor cell, comprising contacting a tumor cell with an adenovirus vector(s) of the invention such that the adenovirus vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell.

In another aspect, methods are provided for detecting a cell which allows the function of a target cell-specific TRE, which comprise contacting a cell in a biological sample with an adenovirus vector(s) of the invention, and detecting replication of the adenovirus vector(s), if any.

5 In another aspect, methods are provided for modifying the genotype of a target cell, comprising contacting the cell with an adenovirus vector as described herein, wherein the adenovirus vector enters the cell.

The present invention provides an adenovirus vector comprising an adenovirus gene, wherein said adenovirus gene is under transcriptional control of a melanocyte-specific TRE. In another embodiment, a melanocyte-specific TRE is human. In another embodiment, a melanocyte-specific TRE comprises a melanocyte-specific promoter and a heterologous enhancer. In other embodiments, a melanocyte-specific TRE comprises a melanocyte-specific promoter. In other embodiments, a melanocyte-specific TRE comprises a melanocyte-specific enhancer and a heterologous promoter. In other 15 embodiments, a melanocyte-specific TRE comprises a melanocyte-specific promoter and a melanocyte-specific enhancer.

In some embodiments, the adenovirus gene under transcriptional control of a melanocyte-specific TRE is an adenovirus gene essential for replication. In some embodiments, the adenoviral gene essential for replication is an early gene. In another 20 embodiment, the early gene is E1A. In another embodiment, the early gene is E1B. In yet another embodiment, both E1A and E1B are under transcriptional control of a melanocyte-specific TRE. In further embodiments, the adenovirus gene essential for replication is E1B, and E1B has a deletion in the 19-kDa region.

In some embodiments, the melanocyte-specific TRE is derived from the 5' flanking 25 region of a tyrosinase gene. In other embodiments, the melanocyte-specific TRE is derived from the 5' flanking region of a tyrosinase related protein-1 gene. In other embodiments, the melanocyte-specific TRE is derived from the 5'-flanking region of a tyrosinase related protein-2 gene. In other embodiments, the melanocyte-specific TRE is derived from the 5' flanking region of a MART-1 gene. In other embodiments, the melanocyte-specific TRE is 30 derived from the 5'-flanking region of a gene which is aberrantly expressed in melanomas.

In other embodiments, the invention provides an adenovirus vector comprising (a) an adenovirus gene under transcriptional control of a melanocyte-specific TRE; and (b) an

E3 region. In some of these embodiments the E3 region is under transcriptional control of a melanocyte-specific TRE.

In another aspect, the invention provides a host cell comprising the melanocyte specific adenovirus vector(s) described herein.

5 In another aspect, the invention provides pharmaceutical compositions comprising a melanocyte specific adenovirus vector(s) described herein.

In another aspect, the invention provides kits which contain a melanocyte adenoviral vector(s) described herein.

10 In another aspect, methods are provided for conferring selective cytotoxicity in target cells (i.e., cells which permit or induce a melanocyte-specific TRE to function), comprising contacting the cells with an adenovirus vector(s) described herein, whereby the vector enters the cell.

15 In another aspect, methods are provided for propagating an adenovirus specific for melanocytes, said method comprising combining an melanocyte specific adenovirus vector(s) described herein with melanocytes, whereby said adenovirus is propagated.

The invention further provides methods of suppressing melanoma cell growth, comprising contacting a melanoma cell with a melanocyte specific adenoviral vector of the invention such that the adenoviral vector enters the melanoma cell and exhibits selective cytotoxicity for the melanoma cell.

20 In another aspect, methods are provided for detecting melanocytes, including melanoma cells, in a biological sample, comprising contacting cells of a biological sample with a melanocyte adenovirus vector(s) described herein, and detecting replication of the adenovirus vector, if any.

25

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic of plasmid construct CP627 as described in Example 1.

Figures 2A-2B is a series of schematic depictions of various adenoviruses described herein.

30 Figure 3 depicts the replication efficiency of different viruses as described in Example 4.

Figures 4A and 4B show viral yield for different liver-specific vectors in different cell types.

Figure 5 is a schematic representation of adenovirus vectors comprising AFP-TRE with and without IRES.

Figure 6 depicts an E3 region.

Figure 7 is a schematic representation of adenovirus vectors described herein.

5 Figure 8 depicts in vivo antitumor activity of CV890 containing an IRES. This figure depicts the results of a HepG2 Xenograph treated with CV790 or CV890.

Figure 9 depicts an ADP nucleotide and amino acid sequence.

Figure 10 depicts an IC<sub>50</sub> isobologram of doxorubicin and CV 890 on Hep3B cells at day 5.

10 Figure 11 depicts in vivo efficacy of CV890 with doxorubicin. Hep3B nude mouse xenografts were grouped (n=6) and treated with CV890 alone ( $1 \times 10^{11}$  particles/dose, iv), doxorubicin alone (10mg/kg, ip), CV890 and doxorubicin combination ( $1 \times 10^{11}$  particles of CV890 through tail vein and 10mg/kg doxorubicin ip), or vehicle control. Tumor size was measured weekly and the tumor volume were normalized as 100% at the day of treatment.

15 Error bars represent the standard error of the mean.

Figure 12 shows the virus yield of CV802, CV882 and CV884 in cell lines.

#### MODES FOR CARRYING OUT THE INVENTION

We have discovered and constructed improved adenovirus vectors comprising co-transcribed first and second genes under transcriptional control of a heterologous, target cell-specific transcriptional regulatory element (TRE), wherein the second gene is under translational control of an internal ribosome entry site (IRES). In one embodiment, the first and second genes are co-transcribed as a single mRNA and the second gene has a mutation in or deletion of its endogenous promoter. In another embodiment, at least one of the genes is an adenovirus gene and in yet another embodiment, both genes are adenovirus genes, including adenovirus genes that are essential for viral replication. The adenovirus vector may comprise a gene that contributes to cytotoxicity (whether direct and/or indirect), and/or causes cell death. An example of an adenovirus gene that contributes to cytotoxicity includes, but is not limited to, the adenovirus death protein gene.

30 In some aspects of the present invention, an adenovirus vector comprising co-transcribed first and second genes under transcriptional control of a target cell-specific TRE, wherein the second gene is under translational control of an IRES, exhibits greater specificity for the target cell than an adenovirus vector comprising a target cell-specific

TRE operably linked to a gene and lacking an IRES. In some embodiments, specificity is conferred by preferential transcription and/or translation of the first and second genes due to the presence of a target cell specific TRE. In other embodiments, specificity is conferred by preferential replication of the adenovirus vectors in target cells due to the target cell-specific TRE driving transcription of a gene essential for replication.

Also disclosed herein are IRES containing adenovirus vectors comprising an adenovirus gene essential for viral replication wherein said essential gene has a mutation in or deletion of its endogenous promoter. In an embodiment disclosed herein, the adenovirus vectors comprise the adenovirus early gene E1A which has a deletion of its endogenous promoter. In another embodiment disclosed herein, the adenovirus vectors comprise the adenovirus early gene E1B which has a deletion of its endogenous promoter. In other embodiments disclosed herein, the 19-kDa region of E1B is deleted.

In another aspect, the adenovirus vectors disclosed herein comprise an adenovirus gene essential for viral replication wherein said essential gene has a mutation in or deletion of its endogenous enhancer. In one embodiment, the adenovirus vector comprises the adenovirus early gene E1A which has a mutation of or deletion in its endogenous promoter. In one embodiment, the adenovirus vector comprises the adenovirus early gene E1A which has a mutation of or deletion in E1A enhancer 1. In a further embodiment, the adenovirus vector comprises the adenovirus early gene E1A which has a mutation of or deletion in its endogenous promoter and a mutation of or deletion in the E1A enhancer. In an additional embodiment, the adenovirus vector comprises the adenovirus early gene E1A which has a mutation of or deletion in its endogenous promoter and the adenovirus early gene E1B which has a mutation of or deletion in its endogenous promoter. In an additional embodiment, the adenovirus vector comprises the adenovirus early gene E1A, which has a mutation of or deletion in its endogenous promoter and a mutation of or deletion in the E1A enhancer I, and the adenovirus early gene E1B which has a mutation of or deletion in its endogenous promoter. In other embodiments disclosed herein, the 19-kDa region of E1B is deleted.

The replication-competent adenovirus vectors of the present invention take advantage of what has been heretofore considered an undesirable aspect of adenovirus vectors, namely their replication and possible concomitant immunogenicity. Runaway infection is prevented due to the cell-specific requirements for viral replication. Without wishing to be bound by any particular theory, it is noted that production of adenovirus

proteins can serve to activate and/or stimulate the immune system, either generally or specifically, toward target cells producing adenoviral proteins. This type of immune stimulation can be an important consideration in the cancer context, where patients are often moderately to severely immunocompromised.

5        The adenovirus vectors of the present invention comprising an intergenic IRES element(s) which links the translation of two or more genes, reflects an improvement over vector constructs which use identical control regions to drive expression of two or more desired genes in that any potential for homologous recombination based on the presence of homologous control regions in the vector is removed. As demonstrated herein, adenovirus  
10      vectors comprising an IRES are stable and in some embodiments provide better specificity than vectors not containing an IRES. Another advantage of an adenovirus vector comprising an intergenic IRES is that the use of an IRES rather than a second TRE may provide additional space in the vector for an additional gene(s) such as a therapeutic gene.

Thus, the adenovirus vectors comprising a second gene under control of an IRES  
15      retain a high level of target cell specificity and remain stable in the target cell.

Accordingly, in one aspect of the invention, the viral vectors disclosed herein comprise at least one IRES within a multicistronic transcript, wherein production of the multicistronic transcript is regulated by a heterologous, target cell-specific TRE. For adenovirus vectors comprising a second gene under control of an IRES, it is preferred that the endogenous  
20      promoter of a gene under translational control of an IRES be deleted so that the endogenous promoter does not interfere with transcription of the second gene. It is preferred that the second gene be in frame with the IRES if the IRES contains an initiation codon. If an initiation codon, such as ATG, is present in the IRES, it is preferred that the initiation codon of the second gene is removed and that the IRES and the second gene are  
25      in frame. Alternatively, if the IRES does not contain an initiation codon or if the initiation codon is removed from the IRES, the initiation codon of the second gene is used. In one embodiment, the adenovirus vectors comprises the adenovirus essential genes, E1A and E1B genes, under the transcriptional control of a heterologous, cell-specific TRE, and an IRES introduced between E1A and E1B. Thus, both E1A and E1B are under common  
30      transcriptional control, and translation of E1B coding region is obtained by virtue of the presence of the IRES. In one embodiment, E1A has its endogenous promoter deleted. In another embodiment, E1A has an endogenous enhancer deleted and in yet an additional embodiment, E1A has its endogenous promoter deleted and E1A enhancer I deleted. In

another embodiment, E1B has its endogenous promoter deleted. In other embodiments disclosed herein, the 19-kDa region of E1B is deleted.

To provide cytotoxicity to target cells, one or more transgenes having a cytotoxic effect may be present in the vector. Additionally, or alternatively, an adenovirus gene that 5 contributes to cytotoxicity and/or cell death, such as the adenovirus death protein (ADP) gene, can be included in the vector, optionally under the selective transcriptional control of a heterologous TRE and optionally under the translational control of an IRES.

Examples of target cells include neoplastic cells, although any cell for which it is desirable and/or tolerable to sustain a cytotoxic activity can be a target cell. By combining 10 an adenovirus vector(s) comprising a target cell-specific TRE with a mixture of target and non-target cells, *in vitro* or *in vivo*, the vector(s) preferentially replicates in the target cells, causing cytotoxic and/or cytolytic effects. Once the target cells are destroyed due to selective cytotoxic and/or cytolytic activity, replication of the vector(s) is significantly reduced, lessening the probability of runaway infection and undesirable bystander effects. 15 *In vitro* cultures can be retained to continually monitor the mixture (such as, for example, a biopsy or other appropriate biological sample) for the presence of the undesirable target cell, *e.g.*, a cancer cell in which the target cell-specific TRE is functional. The adenovirus vectors of the present invention can also be used in *ex vivo* procedures wherein desirable biological samples comprising target cells are removed from the animal, subjected to 20 exposure to an adenovirus vector of the present invention comprising a target cell-specific TRE and then replaced within the animal.

### General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), 25 microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook *et al.*, 1989); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Animal Cell Culture* (R.I. Freshney, ed., 1987); *Methods in Enzymology* (Academic Press, Inc.); *Handbook of Experimental Immunology* (D.M. Wei & 30 C.C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller & M.P. Calos, eds., 1987); *Current Protocols in Molecular Biology* (F.M. Ausubel *et al.*, eds., 1987 and annual updates); *PCR: The Polymerase Chain Reaction*, (Mullis *et al.*, eds., 1994); *Current Protocols in Immunology* (J.E. Coligan *et al.*, eds., 1991 and annual updates).

For techniques related to adenovirus, see, *inter alia*, Felgner and Ringold (1989) *Nature* 337:387-388; Berkner and Sharp (1983) *Nucl. Acids Res.* 11:6003-6020; Graham (1984) *EMBO J.* 3:2917-2922; Bett et al. (1993) *J. Virology* 67:5911-5921; Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806.

5           **Definitions**

As used herein, an "internal ribosome entry site" or "IRES" refers to an element that promotes direct internal ribosome entry to the initiation codon, such as ATG, of a cistron (a protein encoding region), thereby leading to the cap-independent translation of the gene. Jackson RJ, Howell MT, Kaminski A (1990) *Trends Biochem Sci* 15(12):477-83) and Jackson RJ and Kaminski, A. (1995) *RNA* 1(10):985-1000). The present invention encompasses the use of any IRES element which is able to promote direct internal ribosome entry to the initiation codon of a cistron. "Under translational control of an IRES" as used herein means that translation is associated with the IRES and proceeds in a cap-independent manner. Examples of "IRES" known in the art include, but are not limited to IRES obtainable from picornavirus (Jackson et al., 1990, *Trends Biochem Sci* 15(12):477-483); and IRES obtainable from viral or cellular mRNA sources, such as for example, immunoglobulin heavy-chain binding protein (BiP), the vascular endothelial growth factor (VEGF) (Huez et al. (1998) *Mol. Cell. Biol.* 18(11):6178-6190), the fibroblast growth factor 2, and insulin-like growth factor, the translational initiation factor eIF4G, yeast transcription factors TFIID and HAP4. IRES have also been reported in different viruses such as cardiovirus, rhinovirus, aphthovirus, HCV, Friend murine leukemia virus (FrMLV) and Moloney murine leukemia virus (MoMLV). As used herein, "IRES" encompasses functional variations of IRES sequences as long as the variation is able to promote direct internal ribosome entry to the initiation codon of a cistron. In preferred embodiments, the IRES is mammalian. In other embodiments, the IRES is viral or protozoan. In one illustrative embodiment disclosed herein, the IRES is obtainable from encephelomyocarditis virus (ECMV) (commercially available from Novogen, Duke et al. (1992) *J. Virol* 66(3):1602-1609). In another illustrative embodiment disclosed herein, the IRES is from VEGF. Table I and Table II disclose a variety of IRES sequences useful in the present invention.

A "multicistronic transcript" refers to an mRNA molecule which contains more than one protein coding region, or cistron. A mRNA comprising two coding regions is denoted a "bicistronic transcript." The "5'-proximal" coding region or cistron is the coding

region whose translation initiation codon (usually AUG) is closest to the 5'-end of a multicistronic mRNA molecule. A "5'-distal" coding region or cistron is one whose translation initiation codon (usually AUG) is not the closest initiation codon to the 5' end of the mRNA. The terms "5'-distal" and "downstream" are used synonymously to refer to 5 coding regions that are not adjacent to the 5' end of a mRNA molecule.

As used herein, "co-transcribed" means that two (or more) coding regions of polynucleotides are under transcriptional control of single transcriptional control element.

A "gene" refers to a coding region of a polynucleotide. A "gene" may or may not include non-coding sequences and/or regulatory elements.

As used herein, a "transcription response element" or "transcriptional regulatory element", or "TRE" is a polynucleotide sequence, preferably a DNA sequence, which increases transcription of an operably linked polynucleotide sequence in a host cell that allows that TRE to function. A TRE can comprise an enhancer and/or a promoter. A "transcriptional regulatory sequence" is a TRE. A "target cell-specific transcriptional response element" or "target cell-specific TRE" is a polynucleotide sequence, preferably a DNA sequence, which is preferentially functional in a specific type of cell, that is, a target cell. Accordingly, a target cell-specific TRE transcribes an operably linked polynucleotide sequence in a target cell that allows the target cell-specific TRE to function. The term "target cell-specific", as used herein, is intended to include cell type specificity, tissue specificity, developmental stage specificity, and tumor specificity, as well as specificity for a cancerous state of a given target cell. "Target cell-specific TRE" includes cell type-specific and cell status-specific TRE, as well as "composite" TREs. The term "composite TRE" includes a TRE which comprises both a cell type-specific and a cell status-specific TRE. A target cell-specific TRE can also include a heterologous component, including, for example, an SV40 or a cytomegalovirus (CMV) promoter(s). An example of a target cell specific TRE which is tissue specific is a CMV TRE which contains both promoter(s) and enhancer(s).

As described in more detail herein, a target cell-specific TRE can comprise any number of configurations, including, but not limited to, a target cell-specific promoter; and 30 target cell-specific enhancer; a heterologous promoter and a target cell-specific enhancer; a target cell-specific promoter and a heterologous enhancer; a heterologous promoter and a heterologous enhancer; and multimers of the foregoing. The promoter and enhancer components of a target cell-specific TRE may be in any orientation and/or distance from

the coding sequence of interest, as long as the desired target cell-specific transcriptional activity is obtained. Transcriptional activation can be measured in a number of ways known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of mRNA or the protein product of the coding sequence under control of (i.e., operably linked to) the target cell-specific TRE. As discussed herein, a target cell-specific TRE can be of varying lengths, and of varying sequence composition. As used herein, the term "cell status-specific TRE" is preferentially functional, i.e., confers transcriptional activation on an operably linked polynucleotide in a cell which allows a cell status-specific TRE to function, i.e., a cell which exhibits a particular physiological condition, including, but not limited to, an aberrant physiological state. "Cell status" thus refers to a given, or particular, physiological state (or condition) of a cell, which is reversible and/or progressive. The physiological state may be generated internally or externally; for example, it may be a metabolic state (such as in response to conditions of low oxygen), or it may be generated due to heat or ionizing radiation. "Cell status" is distinct from a "cell type", which relates to a differentiation state of a cell, which under normal conditions is irreversible. Generally (but not necessarily), as discussed herein, a cell status is embodied in an aberrant physiological state, examples of which are given below.

A "functional portion" of a target cell-specific TRE is one which confers target cell-specific transcription on an operably linked gene or coding region, such that the operably linked gene or coding region is preferentially expressed in the target cells.

By "transcriptional activation" or an "increase in transcription," it is intended that transcription is increased above basal levels in the target cell (i.e., target cell) by at least about 2 fold, preferably at least about 5 fold, preferably at least about 10 fold, more preferably at least about 20 fold, more preferably at least about 50 fold, more preferably at least about 100 fold, more preferably at least about 200 fold, even more preferably at least about 400 fold to about 500 fold, even more preferably at least about 1000 fold. Basal levels are generally the level of activity (if any) in a non-target cell (i.e., a different cell type), or the level of activity (if any) of a reporter construct lacking a target cell-specific TRE as tested in a target cell line.

A "functionally-preserved variant" of a target cell-specific TRE is a target cell-specific TRE which differs from another target cell-specific TRE, but still retains target cell-specific transcription activity, although the degree of activation may be altered (as

discussed below). The difference in a target cell-specific TRE can be due to differences in linear sequence, arising from, for example, single base mutation(s), addition(s), deletion(s), and/or modification(s) of the bases. The difference can also arise from changes in the sugar(s), and/or linkage(s) between the bases of a target cell-specific TRE. For example, 5 certain point mutations within sequences of TREs have been shown to decrease transcription factor binding and stimulation of transcription. See Blackwood, et al. (1998) *Science* 281:60-63 and Smith et al. (1997) *J. Biol. Chem.* 272:27493-27496. One of skill in the art would recognize that some alterations of bases in and around transcription factor binding sites are more likely to negatively affect stimulation of transcription and cell- 10 specificity, while alterations in bases which are not involved in transcription factor binding are not as likely to have such effects. Certain mutations are also capable of increasing TRE activity. Testing of the effects of altering bases may be performed *in vitro* or *in vivo* by any method known in the art, such as mobility shift assays, or transfecting vectors containing these alterations in TRE functional and TRE non-functional cells. Additionally, one of 15 skill in the art would recognize that point mutations and deletions can be made to a TRE sequence without altering the ability of the sequence to regulate transcription.

As used herein, a TRE derived from a specific gene is referred to by the gene from which it was derived and is a polynucleotide sequence which regulates transcription of an operably linked polynucleotide sequence in a host cell that expresses said gene. For 20 example, as used herein, a "human glandular kallikrein transcriptional regulatory element", or "*hKLK2*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which increases transcription of an operably linked polynucleotide sequence in a host cell that allows an *hKLK2*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses androgen receptor, such as a prostate cell. An 25 *hKLK2*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of an *hKLK2* promoter and/or an *hKLK2* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a "probasin (*PB*) transcriptional regulatory element", or "*PB*-TRE" 30 is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably-linked polynucleotide sequence in a host cell that allows a *PB*-TRE to function, such as a cell (preferably a mammalian cell, more preferably a human cell, even more preferably a prostate cell) that expresses androgen receptor. A *PB*-TRE is

thus responsive to the binding of androgen receptor and comprises at least a portion of a *PB* promoter and/or a *PB* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a “prostate-specific antigen (*PSA*) transcriptional regulatory element”, or “*PSA*-TRE”, or “*PSE*-TRE” is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably linked polynucleotide sequence in a host cell that allows a *PSA*-TRE to function, such as a cell (preferably a mammalian cell, more preferably a human cell, even more preferably a prostate cell) that expresses androgen receptor. A *PSA*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of a *PSA* promoter and/or a *PSA* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a “carcinoembryonic antigen (*CEA*) transcriptional regulatory element”, or “*CEA*-TRE” is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably linked polynucleotide sequence in a host cell that allows a *CEA*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses CEA. The *CEA*-TRE is responsive to transcription factors and/or co-factor(s) associated with CEA-producing cells and comprises at least a portion of the *CEA* promoter and/or enhancer.

As used herein, an “ $\alpha$ -fetoprotein (*AFP*) transcriptional regulatory element”, or “*AFP*-TRE” is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription (of an operably linked polynucleotide sequence) in a host cell that allows an *AFP*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses AFP. The *AFP*-TRE is responsive to transcription factors and/or co-factor(s) associated with AFP-producing cells and comprises at least a portion of the *AFP* promoter and/or enhancer.

As used herein, an “a mucin gene (*MUC*) transcriptional regulatory element”, or “*MUC1*-TRE” is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription (of an operably-linked polynucleotide sequence) in a host cell that allows a *MUC1*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses MUC1. The *MUC1*-TRE is responsive to transcription factors and/or co-factor(s) associated with MUC1-producing cells and comprises at least a portion of the *MUC1* promoter and/or enhancer.

As used herein, a “urothelial cell-specific transcriptional response element”, or “urothelial cell-specific TRE” is polynucleotide sequence, preferably a DNA sequence,

which increases transcription of an operably linked polynucleotide sequence in a host cell that allows a urothelial-specific TRE to function, i.e., a target cell. A variety of urothelial cell-specific TREs are known, are responsive to cellular proteins (transcription factors and/or co-factor(s)) associated with urothelial cells, and comprise at least a portion of a 5 urothelial-specific promoter and/or a urothelial-specific enhancer. Methods are described herein for measuring the activity of a urothelial cell-specific TRE and thus for determining whether a given cell allows a urothelial cell-specific TRE to function.

As used herein, a "melanocyte cell-specific transcriptional response element", or "melanocyte cell-specific TRE" is polynucleotide sequence, preferably a DNA sequence, 10 which increases transcription of an operably linked polynucleotide sequence in a host cell that allows a melanocyte-specific TRE to function, i.e., a target cell. A variety of melanocyte cell-specific TREs are known, are responsive to cellular proteins (transcription factors and/or co-factor(s)) associated with melanocyte cells, and comprise at least a portion of a melanocyte-specific promoter and/or a melanocyte-specific enhancer. 15 Methods are described herein for measuring the activity of a melanocyte cell-specific TRE and thus for determining whether a given cell allows a melanocyte cell-specific TRE to function.

An "E1B 19-kDa region" (used interchangeably with "E1B 19-kDa genomic region") refers to the genomic region of the adenovirus E1B gene encoding the E1B 19-kDa product. According to wild-type Ad5, the E1B 19-kDa region is a 261bp region 20 located between nucleotide 1714 and nucleotide 2244. The E1B 19-kDa region has been described in, for example, Rao *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:7742-7746. The present invention encompasses deletion of part or all of the E1B 19-kDa region as well as 25 embodiments wherein the E1B 19-kDa region is mutated, as long as the deletion or mutation lessens or eliminates the inhibition of apoptosis associated with E1B-19kDa.

As used herein, a target cell-specific TRE can comprise any number of configurations, including, but not limited to, a target cell-specific promoter; a target cell-specific enhancer; a target cell-specific promoter and a target cell-specific enhancer; a target cell-specific promoter and a heterologous enhancer; a heterologous promoter and a 30 target cell-specific enhancer; and multimers of the foregoing. The promoter and enhancer components of a target cell-specific TRE may be in any orientation and/or distance from the coding sequence of interest, as long as the desired target cell-specific transcriptional activity is obtained. Transcriptional activation can be measured in a number of ways

known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of mRNA or the protein product of the coding sequence under control of (i.e., operably linked to) the target cell-specific TRE.

“Replicating preferentially”, as used herein, means that the adenovirus replicates 5 more in a target cell than a non-target cell. Preferably, the adenovirus replicates at a significantly higher rate in target cells than non target cells; preferably, at least about 2-fold higher, preferably, at least about 5-fold higher, more preferably, at least about 10-fold higher, still more preferably at least about 50-fold higher, even more preferably at least about 100-fold higher, still more preferably at least about 400- to 500-fold higher, still 10 more preferably at least about 1000-fold higher, most preferably at least about  $1 \times 10^6$  higher. Most preferably, the adenovirus replicates solely in the target cells (that is, does not replicate or replicates at a very low levels in non-target cells).

As used herein, the term “vector” refers to a polynucleotide construct designed for transduction/transfection of one or more cell types. Vectors may be, for example, “cloning 15 vectors” which are designed for isolation, propagation and replication of inserted nucleotides, “expression vectors” which are designed for expression of a nucleotide sequence in a host cell, or a “viral vector” which is designed to result in the production of a recombinant virus or virus-like particle, or “shuttle vectors”, which comprise the attributes of more than one type of vector.

20 An “adenovirus vector” or “adenoviral vector” (used interchangeably) comprises a polynucleotide construct of the invention. A polynucleotide construct of this invention may be in any of several forms, including, but not limited to, DNA, DNA encapsulated in an adenovirus coat, DNA packaged in another viral or viral-like form (such as herpes simplex, and AAV), DNA encapsulated in liposomes, DNA complexed with polylysine, 25 complexed with synthetic polycationic molecules, conjugated with transferrin, and complexed with compounds such as PEG to immunologically “mask” the molecule and/or increase half-life, and conjugated to a nonviral protein. Preferably, the polynucleotide is DNA. As used herein, “DNA” includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, 30 internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides. For purposes of this invention, adenovirus vectors are replication-competent in a target cell.

The terms "polynucleotide" and "nucleic acid", used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be a 5 oligodeoxynucleoside phosphoramidate (P-NH<sub>2</sub>) or a mixed phosphoramidate-phosphodiester oligomer. Peyrottes et al. (1996) *Nucleic Acids Res.* 24: 1841-8; Chaturvedi et al. (1996) *Nucleic Acids Res.* 24: 2318-23; Schultz et al. (1996) *Nucleic Acids Res.* 24: 2966-73. A phosphorothioate linkage can be used in place of a 10 phosphodiester linkage. Braun et al. (1988) *J. Immunol.* 141: 2084-9; Latimer et al. (1995) *Molec. Immunol.* 32: 1057-1064. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by 15 synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand *de novo* using a DNA polymerase with an appropriate primer. Reference to a polynucleotide sequence (such as referring to a 20 SEQ ID NO) also includes the complement sequence.

The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A 25 polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications included 30 in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to proteins, metal ions, labeling components, other polynucleotides, or a solid support. Preferably, the polynucleotide is DNA. As used herein, "DNA" includes not only bases A, T, C, and G,

but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides.

A polynucleotide or polynucleotide region has a certain percentage (for example, 5 80%, 85%, 90%, or 95%) of "sequence identity" to another sequence means that, when aligned, that percentage of bases are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18. A preferred 10 alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using default parameters, which are as follows: mismatch = 2; open gap = 0; extend gap = 2.

"Under transcriptional control" is a term well understood in the art and indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its 15 being operably (operatively) linked to an element which contributes to the initiation of, or promotes, transcription. "Operably linked" refers to a juxtaposition wherein the elements are in an arrangement allowing them to function.

An "E3 region" (used interchangeably with "E3") is a term well understood in the art and means the region of the adenoviral genome that encodes the E3 products (discussed 20 herein). Generally, the E3 region is located between about 28583 and 30470 of the adenoviral genome. The E3 region has been described in various publications, including, for example, Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199:237-274.

A "portion" of the E3 region means less than the entire E3 region, and as such includes polynucleotide deletions as well as polynucleotides encoding one or more 25 polypeptide products of the E3 region.

As used herein, "cytotoxicity" is a term well understood in the art and refers to a state in which a cell's usual biochemical or biological activities are compromised (i.e., inhibited). These activities include, but are not limited to, metabolism; cellular replication; DNA replication; transcription; translation; uptake of molecules. "Cytotoxicity" includes 30 cell death and/or cytolysis. Assays are known in the art which indicate cytotoxicity, such as dye exclusion, <sup>3</sup>H-thymidine uptake, and plaque assays.

The term "selective cytotoxicity", as used herein, refers to the cytotoxicity conferred by an adenovirus vector of the present invention on a cell which allows or

induces a target cell-specific TRE to function (a target cell) when compared to the cytotoxicity conferred by an adenoviral vector of the present invention on a cell which does not allow a target cell-specific TRE to function (a non-target cell). Such cytotoxicity may be measured, for example, by plaque assays, by reduction or stabilization in size of a tumor comprising target cells, or the reduction or stabilization of serum levels of a marker characteristic of the tumor cells, or a tissue-specific marker, e.g., a cancer marker.

5 In the context of adenovirus, a "heterologous polynucleotide" or "heterologous gene" or "transgene" is any polynucleotide or gene that is not present in wild-type adenovirus. Preferably, the transgene will also not be expressed or present in the target cell prior to introduction by the adenovirus vector. Examples of preferred transgenes are 10 provided below.

In the context of adenovirus, a "heterologous" promoter or enhancer is one which is not associated with or derived from an adenovirus gene.

15 In the context of adenovirus, an "endogenous" promoter, enhancer, or TRE is native to or derived from adenovirus. In the context of promoter, an "inactivation" means that there is a mutation of or deletion in part or all of the endogenous promoter, i.e., a modification or alteration of the endogenous promoter, such as, for example, a point mutation or insertion, which disables the function of the promoter.

20 In the context of a target cell-specific TRE, a "heterologous" promoter or enhancer is one which is derived from a gene other than the gene from which a reference target cell-specific TRE is derived.

25 "Suppressing" tumor growth indicates a growth state that is curtailed when compared to growth without contact with, i.e., transfection by, an adenoviral vector described herein. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a  $^3\text{H}$ -thymidine incorporation assay, or counting tumor cells.

30 "Suppressing" tumor cell growth means any or all of the following states: slowing, delaying, and stopping tumor growth, as well as tumor shrinkage.

As used herein, the terms "neoplastic cells", "neoplasia", "tumor", "tumor cells", "cancer" and "cancer cells", (used interchangeably) refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation (i.e., de-regulated cell division). Neoplastic cells can be malignant or benign.

A "host cell" includes an individual cell or cell culture which can be or has been a recipient of an adenoviral vector(s) of this invention. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate 5 mutation and/or change. A host cell includes cells transfected or infected *in vivo* or *in vitro* with an adenoviral vector of this invention.

"Replication" and "propagation" are used interchangeably and refer to the ability of an adenovirus vector of the invention to reproduce or proliferate. These terms are well understood in the art. For purposes of this invention, replication involves production of 10 adenovirus proteins and is generally directed to reproduction of adenovirus. Replication can be measured using assays standard in the art and described herein, such as a burst assay or plaque assay. "Replication" and "propagation" include any activity directly or indirectly involved in the process of virus manufacture, including, but not limited to, viral gene expression; production of viral proteins, nucleic acids or other components; packaging of 15 viral components into complete viruses; and cell lysis.

An "ADP coding sequence" is a polynucleotide that encodes ADP or a functional fragment thereof. In the context of ADP, a "functional fragment" of ADP is one that exhibits cytotoxic activity, especially cell lysis, with respect to adenoviral replication. Ways to measure cytotoxic activity are known in the art and are described herein.

20 A polynucleotide that "encodes" an ADP polypeptide is one that can be transcribed and/or translated to produce an ADP polypeptide or a fragment thereof. The anti-sense strand of such a polynucleotide is also said to encode the sequence.

An "ADP polypeptide" is a polypeptide containing at least a portion, or region, of the amino acid sequence of an ADP and which displays a function associated with ADP, 25 particularly cytotoxicity, more particularly, cell lysis. As discussed herein, these functions can be measured using techniques known in the art. It is understood that certain sequence variations may be used, due to, for example, conservative amino acid substitutions, which may provide ADP polypeptides.

"Androgen receptor," or AR, as used herein refers to a protein whose function is to 30 specifically bind to androgen and, as a consequence of the specific binding, recognize and bind to an androgen response element (ARE), following which the AR is capable of regulating transcriptional activity. The AR is a nuclear receptor that, when activated, binds to cellular androgen-responsive element(s). In normal cells the AR is activated by

androgen, but in non-normal cells (including malignant cells) the AR may be activated by non-androgenic agents, including hormones other than androgens. Encompassed in the term "androgen receptor" are mutant forms of an androgen receptor, such as those characterized by amino acid additions, insertions, truncations and deletions, as long as the 5 function is sufficiently preserved. Mutants include androgen receptors with amino acid additions, insertions, truncations and deletions, as long as the function is sufficiently preserved. In this context, a functional androgen receptor is one that binds both androgen and, upon androgen binding, an ARE.

A polynucleotide sequence that is "depicted in" a SEQ ID NO means that the 10 sequence is present as an identical contiguous sequence in the SEQ ID NO. The term encompasses portions, or regions of the SEQ ID NO as well as the entire sequence contained within the SEQ ID NO.

A "biological sample" encompasses a variety of sample types obtained from an individual and can be used in a diagnostic or monitoring assay. The definition 15 encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom, and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides. The term "biological sample" 20 encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples.

An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, farm animals, sport animals, rodents, primates, and pets.

25 An "effective amount" is an amount sufficient to effect beneficial or desired results, including clinical results. An effective amount can be administered in one or more administrations. For purposes of this invention, an effective amount of an adenoviral vector is an amount that is sufficient to palliate, ameliorate, stabilize, reverse, slow or delay the progression of the disease state.

30 A given TRE is "derived from" a given gene if it is associated with that gene in nature.

"Expression" includes transcription and/or translation.

As used herein, the term "comprising" and its cognates are used in their inclusive sense; that is, equivalent to the term "including" and its corresponding cognates.

"A," "an" and "the" include plural references unless the context clearly dictates otherwise.

5        *Internal Ribosome Entry Site (IRES)*

IRES elements were first discovered in picornavirus mRNAs (Jackson RJ, Howell MT, Kaminski A (1990) *Trends Biochem Sci* 15(12):477-83) and Jackson RJ and Kaminski, A. (1995) *RNA* 1(10):985-1000). The present invention provides improved adenovirus vectors comprising co-transcribed first and second genes under transcriptional 10 control of a heterologous, target cell-specific TRE, and wherein the second gene (i.e., coding region) is under translational control of an internal ribosome entry site (IRES). Any IRES may be used in the adenovirus vectors of the invention, as long as they exhibit requisite function in the vectors. Examples of IRES which can be used in the present invention include those provided in Table I and referenced in Table II. Examples of IRES 15 elements include the encephelomyocarditis virus (EMCV) which is commercially available from Novagen (Duke et al. (1992) *J. Virol* 66(3):1602-9) the sequence for which is depicted in Table 1 (SEQ ID NO:1). Another example of an IRES element disclosed herein is the VEGF IRES (Huez et al. (1998) *Mol Cell Biol* 18(11):6178-90). This IRES has a short segment and the sequence is depicted in Table 1 (SEQ ID NO:2).

20        The IRES promotes direct internal ribosome entry to the initiation codon of a downstream cistron, leading to cap-independent translation. Thus, the product of a downstream cistron can be expressed from a bicistronic (or multicistronic) mRNA, without requiring either cleavage of a polyprotein or generation of a monocistronic mRNA. Therefore, in one illustrative embodiment of the present invention, an adenovirus vector 25 comprising E1B under translational control of an IRES allows translation of E1B from a bicistronic E1A-E1B mRNA under control of a target cell-specific TRE. Figure 7 provides a schematic representation of adenovirus constructs of the present invention.

Internal ribosome entry sites are approximately 450 nucleotides in length and are characterized by moderate conservation of primary sequence and strong conservation of 30 secondary structure. The most significant primary sequence feature of the IRES is a pyrimidine-rich site whose start is located approximately 25 nucleotides upstream of the 3' end of the IRES. *See Jackson et al. (1990).*

Three major classes of picornavirus IRES have been identified and characterized: (1) the cardio- and aphthovirus class (for example, the encephelomyocarditis virus, Jang et al. (1990) *Gene Dev* 4:1560-1572); (2) the enter- and rhinovirus class (for example, polioviruses, Borman et al. (1994) *EMBO J.* 13:314903157); and (3) the hepatitis A virus (HAV) class, Glass et al. (1993) *Virol* 193:842-852). For the first two classes, two general principles apply. First, most of the 450-nucleotide sequence of the IRES functions to maintain particular secondary and tertiary structures conducive to ribosome binding and translational initiation. Second, the ribosome entry site is an AUG triplet located at the 3' end of the IRES, approximately 25 nucleotides downstream of a conserved oligopyrimidine tract. Translation initiation can occur either at the ribosome entry site (cardioviruses) or at the next downstream AUG (entero/rhinovirus class). Initiation occurs at both sites in aphthoviruses.

HCV and pestiviruses such as bovine viral diarrhea virus (BVDV) or classical swine fever virus (CSFV) have 341 nt and 370 nt long 5'-UTR respectively. These 5'-UTR fragments form similar RNA secondary structures and can have moderately efficient IRES function (Tsukiyama-Kohara et al. (1992) *J. Virol.* 66:1476-1483; Frolov I et al., (1998) *RNA* 4:1418-1435). Table I depicts the 5'-UTR region from HCV genome sequence (GenBank accession D14853).

Leishmania RNA virus 1 (LRV1) is a double-stranded RNA virus. Its 128 nt long 5'-UTR has IRES activity to facilitate the cap-independent translation, (Maga et al. (1995) *Mol Cell Biol* 15:4884-4889). This fragment also forms conserved stemloop secondary structure and at least the front part is essential.

Recent studies showed that both Friend-murine leukemia virus (MLV) 5'-UTR and rat retrotransposon virus-like 30S (VL30) sequences contain IRES structure of retroviral origin (Torrent et al. (1996) *Hum Gene Ther* 7:603-612). These fragments are also functional as packing signal when used in retrovirus derived vectors. Studies of avian reticuloendotheliosis virus type A (REV-A) show that its IRES maps downstream of the packaging/dimerization (E/DLS) sequence and the minimal IRES sequence appears to be within a 129 nt fragment (452-580) of the 5' leader, immediately upstream of the gag AUG codon (Lopez-Lastra et al. (1997) *Hum Gene Ther* 8:1855-1865).

In eukaryotic cells, translation is normally initiated by the ribosome scanning from the capped mRNA 5' end, under the control of initiation factors. However, several cellular

mRNAs have been found to have IRES structure to mediate the cap-independent translation (van der Velde, et al. (1999) *Int J Biochem Cell Biol.* 31:87-106). Examples are immunoglobulin heavy-chain binding protein (BiP) (Macejak et al. (1991) *Nature* 353:90-94), antennapedia mRNA of Drosophila (Oh et al. (1992) *Gene and Dev* 6:1643-1653), fibroblast growth factor-2 (FGF-2) (Vagner et al. (1995) *Mol Cell Biol* 15:35-44), platelet-derived growth factor B (PDGF-B) (Bernstein et al. (1997) *J Biol Chem* 272:9356-9362), insulin-like growth factor II (Teerink et al. (1995) *Biochim Biophys Acta* 1264:403-408), and the translation initiation factor eIF4G (Gan et al. (1996) *J Biol Chem* 271:623-626). Table 1 depicts the 5'-noncoding region for BiP and PDGF. Recently, 10 vascular endothelial growth factor (VEGF) was also found to have IRES element (Stein et al. (1998) *Mol Cell Biol* 18:3112-3119; Huez et al. (1998) *Mol Cell Biol* 18:6178-6190).

Apart from the oligopyrimidine tract, nucleotide sequence *per se* does not appear to be important for IRES function. Without wishing to be bound by theory, a possible explanation for the function of an IRES is that it forms secondary and/or tertiary structures 15 which orient particular single-stranded regions of its sequence in a three-dimensional configuration that is conducive to interaction with a mammalian ribosome (either ribosomal protein and/or ribosomal RNA components) and/or initiation factor(s) and/or RNA binding proteins which interact with ribosomes and/or initiation factors. It is also possible that the three-dimensional structure of the IRES is determined or stabilized by one or more RNA-20 binding proteins. Thus it is possible to devise synthetic IRES sequences having similar single-stranded regions in a similar three-dimensional configuration.

In certain cases, one or more *trans*-acting cellular proteins may be required for IRES function. For example, the HAV and entero/rhinovirus IRESes function inefficiently 25 *in vitro* in reticulocyte lysates. Supplementation of a reticulocyte lysate with a cytoplasmic extract from HeLa, Krebs II ascites, or L-cells restores activity of entero/rhinovirus IRESes. See, for example, Brown et al. (1979) *Virology* 97:396-405; and Dorner et al. (1984) *J. Virol.* 50:507-514. Activity of the HAV IRES *in vitro* is stimulated by liver cytoplasmic extracts. Glass et al. (1993) *Virology* 193:1047-1050. These observations indicate that cell-specific translational regulation can be achieved through the use of a cell-30 specific IRES. Furthermore, coordinated cell-specific transcriptional and translational regulatory elements can be included in a vector to further increase cell specificity of viral replication. For example, the combination of an AFP-TRE and a HAV-IRES can be used to direct preferential replication of a vector in hepatic cells. Thus, in one illustrative

embodiment, a vector comprises an AFP-TRE regulating the transcription of a bicistronic E1A-E1B mRNA in which E1B translation is regulated by an ECMV IRES. In another illustrative embodiment, the vector comprises a probasin-TRE regulating the transcription of a bicistronic E1A-E1B mRNA in which E1B translation is regulated by an ECMV IRES.

5 In yet another illustrative embodiment, a vector comprises a CMV-TRE regulating the transcription of a bicistronic E1A-E1B mRNA in which E1B translation is regulated by an ECMV IRES.

Examples of IRES which can be used in the present invention include those provided in Table 1 and Table 2. An IRES sequence which may be used in the present 10 invention may be tested as follows. A test vector is produced having a reporter gene, such as luciferase, for example, placed under translational control of an IRES to be tested. A desired cell type is transfected with the vector containing the desired IRES-reporter gene and an assay is performed to detect the presence of the reporter gene. In one illustrative example, the test vector comprises a co-transcribed chloramphenicol transferase (CAT) and 15 luciferase encoding gene transcriptionally driven by a CMV promoter wherein the luciferase encoding gene is translationally driven by an IRES to be tested. Host cells are transiently transfected with the test vector by means known to those of skill in the art and assayed for the presence of luciferase.

IRES may be prepared using standard recombinant and synthetic methods known in 20 the art, and as described in the Examples. For cloning convenience, restriction sites may be engineered into the ends of the IRES fragments to be used.

#### *Transcriptional response elements (TREs)*

The adenovirus vectors of the invention comprise target cell specific TREs which direct preferential expression of an operatively linked gene (or genes) in a particular target 25 cell. A TRE can be tissue-specific, tumor-specific, developmental stage-specific, cell status specific, etc., depending on the type of cell present in the tissue or tumor.

Cell- and tissue-specific transcriptional regulatory elements, as well as methods for their identification, isolation, characterization, genetic manipulation and use for regulation of operatively linked coding sequences, are well known in the art. A TRE can be derived 30 from the transcriptional regulatory sequences of a single gene, or sequences from different genes can be combined to produce a functional TRE. A cell-specific TRE is preferentially functional in a limited population (or type) of cells, e.g., prostate cells or liver cells.

Accordingly, in some embodiments, the TRE used is preferentially functional in any of the

following cell types: prostate; liver; breast; urothelial cells (bladder); colon; lung; ovarian; pancreas; stomach; and uterine. In other embodiments, in accordance with cell status, the TRE is functional in or during: low oxygen conditions (hypoxia); certain stages of cell cycle, such as S phase; elevated temperature; ionizing radiation.

5 As is known in the art, activity of TREs can be inducible. Inducible TREs generally exhibit low activity in the absence of inducer, and are up-regulated in the presence of inducer. Inducers include, for example, nucleic acids, polypeptides, small molecules, organic compounds and/or environmental conditions such as temperature, pressure or hypoxia. Inducible TREs may be preferred when expression is desired only at certain times  
10 or at certain locations, or when it is desirable to titrate the level of expression using an inducing agent. For example, transcriptional activity from the *PSE*-TRE, *PB*-TRE and *hKLK2*-TRE is inducible by androgen, as described herein and in PCT/US98/04080. Accordingly, in one embodiment of the present invention, an adenovirus vector comprises an inducible heterologous TRE.

15 TRE multimers are also useful in the disclosed vectors. For example, a TRE can comprise a tandem series of at least two, at least three, at least four, or at least five promoter fragments. Alternatively, a TRE can comprise one or more promoter regions along with one or more enhancer regions. TRE multimers can also comprise promoter and/or enhancer sequences from different genes. The promoter and enhancer components  
20 of a TRE can be in any orientation with respect to each other and can be in any orientation and/or any distance from the coding sequence of interest, as long as the desired cell-specific transcriptional activity is obtained.

The disclosed vectors are designed such that replication is preferentially enhanced in target cells in which the TRE(s) is (are) functional. More than one TRE can be present  
25 in a vector, as long as the TREs are functional in the same target cell. However, it is important to note that a given TRE can be functional in more than one type of target cell. For example, the CEA-TRE functions in, among other cell types, gastric cancer cells, colorectal cancer cells, pancreatic cancer cells and lung cancer cells.

30 A TRE for use in the present vectors may or may not comprise a silencer. The presence of a silencer (*i.e.*, a negative regulatory element known in the art) can assist in shutting off transcription (and thus replication) in non-target cells. Thus, presence of a silencer can confer enhanced cell-specific vector replication by more effectively preventing

replication in non-target cells. Alternatively, lack of a silencer may stimulate replication in target cells, thus conferring enhanced target cell-specificity.

As is readily appreciated by one skilled in the art, a TRE is a polynucleotide sequence, and, as such, can exhibit function over a variety of sequence permutations.

5 Methods of nucleotide substitution, addition, and deletion are known in the art, and readily-available functional assays (such as the CAT or luciferase reporter gene assay) allow one of ordinary skill to determine whether a sequence variant exhibits requisite cell-specific transcription regulatory function. Hence, functionally preserved variants of TREs, comprising nucleic acid substitutions, additions, and/or deletions, can be used in the vectors disclosed herein. Accordingly, variant TREs retain function in the target cell but need not exhibit maximal function. In fact, maximal transcriptional activation activity of a TRE may not always be necessary to achieve a desired result, and the level of induction afforded by a fragment of a TRE may be sufficient for certain applications. For example, if used for treatment or palliation of a disease state, less-than-maximal responsiveness may be

10 sufficient if, for example, the target cells are not especially virulent and/or the extent of disease is relatively confined.

15

Certain base modifications may result in enhanced expression levels and/or cell-specificity. For example, nucleic acid sequence deletions or additions within a TRE can move transcription regulatory protein binding sites closer or farther away from each other than they exist in their normal configuration, or rotate them so they are on opposite sides of the DNA helix, thereby altering spatial relationship among TRE-bound transcription factors, resulting in a decrease or increase in transcription, as is known in the art. Thus, while not wishing to be bound by theory, the present disclosure contemplates the possibility that certain modifications of a TRE will result in modulated expression levels as directed by the TRE, including enhanced cell-specificity. Achievement of enhanced expression levels may be especially desirable in the case of more aggressive forms of neoplastic growth, and/or when a more rapid and/or aggressive pattern of cell killing is warranted (for example, in an immunocompromised individual).

20

25

Transcriptional activity directed by a TRE (including both inhibition and enhancement) can be measured in a number of ways known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of mRNA and/or of a protein product encoded by the sequence under control of (*i.e.*, operably linked to) a TRE.

30

As discussed herein, a TRE can be of varying lengths, and of varying sequence composition. The size of a heterologous TRE will be determined in part by the capacity of the viral vector, which in turn depends upon the contemplated form of the vector (see *infra*). Generally minimal sizes are preferred for TREs, as this provides potential room for 5 insertion of other sequences which may be desirable, such as transgenes (discussed *infra*) and/or additional regulatory sequences. In a preferred embodiment, such an additional regulatory sequence is an IRES. However, if no additional sequences are contemplated, or if, for example, an adenoviral vector will be maintained and delivered free of any viral packaging constraints, larger TRE sequences can be used as long as the resultant adenoviral 10 vector remains replication-competent.

An adenoviral vector can be packaged with extra sequences totaling up to about 5% of the genome size, or approximately 1.8 kb, without requiring deletion of viral sequences. If non-essential sequences are removed from the adenovirus genome, an additional 4.6 kb of insert can be tolerated (*i.e.*, for a total insertion capacity of about 6.4 kb). Examples of 15 non-essential adenoviral sequences that can be deleted are E3, and E4 sequences other than those which encode E4 ORF6.

To minimize non-specific replication, endogenous (*e. g.*, adenovirus) TREs are preferably removed from the vector. Besides facilitating target cell-specific replication, removal of endogenous TREs also provides greater insert capacity in a vector, which may 20 be of special concern if an adenoviral vector is to be packaged within a virus particle. Even more importantly, deletion of endogenous TREs prevents the possibility of a recombination event whereby a heterologous TRE is deleted and the endogenous TRE assumes transcriptional control of its respective adenovirus coding sequences (thus allowing non-specific replication). In one embodiment, an adenoviral vector is constructed such that the 25 endogenous transcription control sequences of adenoviral genes are deleted and replaced by one or more heterologous TREs. However, endogenous TREs can be maintained in the adenovirus vector(s), provided that sufficient cell-specific replication preference is preserved. These embodiments are constructed by inserting heterologous TREs between an 30 endogenous TRE and a replication gene coding segment. Requisite cell-specific replication preference is determined by conducting assays that compare replication of the adenovirus vector in a cell which allows function of the heterologous TREs with replication in a cell which does not.

Generally, a TRE will increase replication of a vector in a target cell by at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold more preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at least about 100-fold, more preferably at least about 200-fold, even more preferably at least about 400- to about 500-fold, even more preferably at least about 1000-fold, compared to basal levels of replication in the absence of a TRE. The acceptable differential can be determined empirically (by measurement of mRNA levels using, for example, RNA blot assays, RNase protection assays or other assays known in the art) and will depend upon the anticipated use of the vector and/or the desired result.

10       Replication-competent adenovirus vectors directed at specific target cells can be generated using TREs that are preferentially functional in a target cell. In one embodiment of the present invention, the target cell is a tumor cell. Non-limiting examples of tumor cell-specific heterologous TREs, and their respective target cells, include: probasin (PB), target cell, prostate cancer (PCT/US98/04132);  $\alpha$ -fetoprotein (*AFP*), target cell liver cancer (PCT/US98/04084); mucin-like glycoprotein DF3 (*MUC1*), target cell, breast carcinoma (PCT/US98/04080); carcinoembryonic antigen (*CEA*), target cells, colorectal, gastric, pancreatic, breast, and lung cancers (PCT/US98/04133); plasminogen activator urokinase (*uPA*) and its receptor gene, target cells, breast, colon, and liver cancers (PCT/US98/04080); *E2F1* (cell cycle S-phase specific promoter); target cell, tumors with disrupted retinoblastoma gene function, and *HER-2/neu* (*c-erbB2/neu*), target cell, breast, ovarian, stomach, and lung cancers (PCT/US98/04080); tyrosinase, target cell, melanoma cells as described herein and uroplakins, target cell, bladder cells as described herein. Methods for identification, isolation, characterization and utilization of additional target cell-specific TREs are readily available to those of skill in the art.

25       In addition, tumor-specific TREs can be used in conjunction with tissue-specific TREs from the following exemplary genes (tissue in which the TREs are specifically functional are in parentheses): hypoxia responsive element, vascular endothelial growth factor receptor (endothelium), albumin (liver), factor VII (liver), fatty acid synthase (liver), Von Willebrand factor (brain endothelium), alpha-actin and myosin heavy chain (both in smooth muscle), synthetase I (small intestine)  $\text{Na}^+ \text{-K}^+ \text{-Cl}^-$  transporter (kidney). Additional tissue-specific TREs are known in the art.

In one embodiment of the present invention, a target cell-specific, heterologous TRE is tumor cell-specific. A vector can comprise a single tumor cell-specific TRE or

multiple heterologous TREs which are tumor cell-specific and functional in the same cell. In another embodiment, a vector comprises one or more heterologous TREs which are tumor cell-specific and additionally comprises one or more heterologous TREs which are tissue specific, whereby all TREs are functional in the same cell.

5                   Prostate-specific TREs

In one embodiment, adenovirus vectors comprise heterologous TREs that are prostate cell specific. For example, TREs that function preferentially in prostate cells and can be used to target adenovirus replication to prostate neoplasia, include, but are not limited to, TREs derived from the prostate-specific antigen gene (*PSA*-TRE) (Henderson 10 U.S. Patent No. 5,698,443); the glandular kallikrein-1 gene (from the human gene, *hKLK2*-TRE) (PCT US98/16312), and the probasin gene (*PB*-TRE) (PCT/US98/04132). All three of these genes are preferentially expressed in prostate cells and their expression is androgen-inducible. Generally, expression of genes responsive to androgen induction is mediated by an androgen receptor (AR).

15                   Prostate-specific Antigen (PSA)

PSA is synthesized exclusively in prostatic epithelial cells and is synthesized in these cells whether they are normal, hyperplastic, or malignant. This tissue-specific expression of PSA has made it an excellent biomarker for benign prostatic hyperplasia (BPH) and prostatic carcinoma (CaP). Normal serum levels of PSA are typically below 5 20 ng/ml, with elevated levels indicative of BPH or CaP. Lundwall *et al.* (1987) *FEBS Lett.* 214:317; Lundwall (1989) *Biochem. Biophys. Res. Comm.* 161:1151; and Riegermann *et al.* (1991) *Molec. Endocrin.* 5:1921.

The region of the *PSA* gene that provides androgen-dependent cell specificity, particularly in prostate cells, involves approximately 6.0 kilobases (kb). Schuur *et al.* 25 (1996) *J. Biol. Chem.* 271:7043-7051. An enhancer region of approximately 1.5 kb in humans is located between nt -5322 and nt -3739, relative to the transcription start site of the *PSA* gene. Within these enhancer sequences is an androgen response element (ARE) a sequence which binds androgen receptor. The sequence coordinates of the *PSA* promoter are from about nt -540 to nt +8 relative to the transcription start site. Juxtapositioning of 30 the enhancer and promoter yields a fully functional, minimal prostate-specific TRE (*PSA*-TRE). Other portions of this approximately 6.0 kb region of the *PSA* gene can be used in the vectors described herein, as long as requisite functionality is maintained.

*Human glandular Kallikrein (hKLK2)*

Human glandular kallikrein (*hKLK2*, encoding the hK2 protein) is expressed exclusively in the prostate and its expression is up-regulated by androgens, primarily through transcriptional activation. Wolf *et al.* (1992) *Molec. Endocrinol.* 6:753-762; 5 Morris (1989) *Clin. Exp. Pharm. Physiol.* 16:345-351; Qui *et al.* (1990) *J. Urol.* 144:1550-1556; and Young *et al.* (1992) *Biochem.* 31:818-824. The levels of hK2 found in various tumors and in the serum of patients with prostate cancer indicate that hK2 antigen may be a significant marker for prostate cancer. Charlesworth *et al.* (1997) *Urology* 49:487-493. Expression of hK2 has been detected in each of 257 radical prostatectomy specimens 10 analyzed. Darson *et al.* (1997) *Urology* 49:857-862. The intensity and extent of hK2 expression, detected using specific antibodies, was observed to increase from benign epithelium to high-grade prostatic intraepithelial neoplasia (PIN) and adenocarcinoma.

The activity of the *hKLK2* promoter has been described and a region up to nt -2256 relative to the transcription start site was previously disclosed. Schedlich *et al.* (1987) *DNA* 15 *6*:429-437. The *hKLK2* promoter is androgen responsive and, in plasmid constructs wherein the promoter alone controls the expression of a reporter gene, expression of the reporter gene is increased approximately 10-fold in the presence of androgen. Murtha *et al.* (1993) *Biochem.* 32:6459-6464. *hKLK2* enhancer activity is found within a polynucleotide sequence approximately nt -12,014 to nt -2257 relative to the start of transcription and, 20 when this sequence is operably linked to an *hKLK2* promoter and a reporter gene, transcription of operably-linked sequences in prostate cells increases in the presence of androgen to levels approximately 30-fold to approximately 100-fold greater than the level of transcription in the absence of androgen. This induction is generally independent of the orientation and position of the enhancer sequences. Enhancer activity has also been 25 demonstrated in the following regions (all relative to the transcription start site): about nt -3993 to about nt -3643, about nt -4814 to about nt -3643, about nt -5155 to about nt -3387, about nt -6038 to about nt -2394.

Thus, a *hKLK2* enhancer can be operably linked to an *hKLK2* promoter or a heterologous promoter to form a *hKLK2* transcriptional regulatory element (*hKLK2*-TRE). 30 A *hKLK2*-TRE can then be operably linked to a heterologous polynucleotide to confer *hKLK2*-TRE-specific transcriptional regulation on the linked gene, thus increasing its expression.

*Probasin*

The rat probasin (*PB*) gene encodes an androgen and zinc-regulated protein first characterized in the dorsolateral prostate of the rat. Dodd *et al.* (1983) *J. Biol. Chem.* 258:10731-10737; Matusik *et al.* (1986) *Biochem. Cell. Biol.* 64:601-607; and Sweetland *et al.* (1988) *Mol. Cell. Biochem.* 84:3-15. The dorsolateral lobes of the murine prostate are considered the most homologous to the peripheral zone of the human prostate, where approximately 68% of human prostate cancers are thought to originate.

A *PB*-TRE has been shown to exist in an approximately 0.5 kb fragment of sequence upstream of the probasin coding sequence, from about nt -426 to about nt +28 relative to the transcription start site. This minimal promoter sequence from the *PB* gene appears to provide sufficient information to direct prostate-specific developmental- and hormone -regulated expression of an operably linked heterologous gene in transgenic mice. Greenberg *et al.* (1994) *Mol. Endocrinol.* 8:230-239.

Alpha-fetoprotein

$\alpha$ -fetoprotein (AFP) is an oncofetal protein, the expression of which is primarily restricted to developing tissues of endodermal origin (yolk sac, fetal liver, and gut), although the level of its expression varies greatly depending on the tissue and the developmental stage. AFP is of clinical interest because the serum concentration of AFP is elevated in a majority of hepatoma patients, with high levels of AFP found in patients with advanced disease. High serum AFP levels in patients appear to be due to AFP expression in hepatocellular carcinoma (HCC), but not in surrounding normal liver. Thus, expression of the AFP gene appears to be characteristic of hepatoma cells. An AFP-TRE is described in for example PCT/US98/04084.

According to published reports, the AFP-TRE is responsive to cellular proteins (transcription factors and/or co-factor(s)) associated with AFP-producing cells, such as AFP-binding protein (see, for example, U.S. Pat. No. 5,302,698) and comprises at least a portion of an AFP promoter and/or an AFP enhancer. Cell-specific TREs from the *AFP* gene have been identified. For example, the cloning and characterization of human AFP-specific enhancer activity is described in Watanabe *et al.* (1987) *J. Biol. Chem.* 262:4812-4818. A 5' *AFP* regulatory region (containing the promoter, putative silencer, and enhancer) is contained within approximately 5 kb upstream from the transcription start site.

Within the AFP regulatory region, a human *AFP* enhancer region is located between about nt -3954 and about nt -3335, relative to the transcription start site of the *AFP*

gene. The human *AFP* promoter encompasses a region from about nt -174 to about nt +29. Juxtapositioning of these two genetic elements, yields a fully functional *AFP*-TRE. Ido *et al.* (1995) *Cancer Res.* 55:3105-3109 describe a 259 bp promoter fragment (nt -230 to nt +29) that is specific for expression in HCC cells. The *AFP* enhancer, located between 5 nt -3954 and nt -3335 relative to the transcription start site, contains two regions, denoted A and B. The promoter region contains typical TATA and CAAT boxes. Preferably, the *AFP*-TRE contains at least one enhancer region. More preferably, the *AFP*-TRE contains both enhancer regions.

Suitable target cells for vectors containing *AFP*-TREs are any cell type that allow 10 an *AFP*-TRE to function. Preferred are cells that express or produce AFP, including, but not limited to, tumor cells expressing AFP. Examples of such cells are hepatocellular carcinoma (HCC) cells, gonadal and other germ cell tumors (especially endodermal sinus tumors), brain tumor cells, ovarian tumor cells, acinar cell carcinoma of the pancreas (Kawamoto *et al.* (1992) *Hepatogastroenterology* 39:282-286), primary gall bladder tumor 15 (Katsuragi *et al.* (1989) *Rinsko Hoshasen* 34:371-374), uterine endometrial adenocarcinoma cells (Koyama *et al.* (1996) *Jpn. J. Cancer Res.* 87:612-617), and any metastases of the foregoing (which can occur in lung, adrenal gland, bone marrow, and/or spleen). In some cases, metastatic disease to the liver from certain pancreatic and stomach cancers produce AFP. Especially preferred as target cells for an *AFP*-TRE are 20 hepatocellular carcinoma cells and any of their metastases.

AFP production can be measured (and hence AFP-producing cells can be identified) using immunoassays standard in the art, such as RIA, ELISA or protein immunoblotting (Western blots) to determine levels of AFP protein production; and/or RNA blotting (Northern blots) to determine AFP mRNA levels. Alternatively, such cells can be 25 identified and/or characterized by their ability to activate transcriptionally an *AFP*-TRE (*i.e.*, allow an *AFP*-TRE to function).

See also co-owned PCT W098/39465 regarding *AFP*-TREs. As described in more detail therein, an *AFP*-TRE can comprise any number of configurations, including, but not limited to, an *AFP* promoter; an *AFP* enhancer; an *AFP* promoter and an *AFP* enhancer; an 30 *AFP* promoter and a heterologous enhancer; a heterologous promoter and an *AFP* enhancer; and multimers of the foregoing. The promoter and enhancer components of an *AFP*-TRE can be in any orientation and/or distance from the coding sequence of interest, as long as the desired *AFP* cell-specific transcriptional activity is obtained. An adenovirus

vector of the present invention can comprise an AFP-TRE endogenous silencer element or the AFP-TRE endogenous silencer element can be deleted.

Urokinase Plasminogen Activator

The protein urokinase plasminogen activator (uPA) and its cell surface receptor, 5 urokinase plasminogen activator receptor (uPAR), are expressed in many of the most frequently-occurring neoplasms and appear to represent important proteins in cancer metastasis. Both proteins are implicated in breast, colon, prostate, liver, renal, lung and ovarian cancer. Sequence elements that regulate uPA and uPAR transcription have been extensively studied. Riccio *et al.* (1985) *Nucleic Acids Res.* 13:2759-2771; Cannio *et al.* 10 (1991) *Nucleic Acids Res.* 19:2303-2308.

Carcinoembryonic antigen (CEA)

CEA is a 180,000 Dalton, tumor-associated, glycoprotein antigen present on 15 endodermally-derived neoplasms of the gastrointestinal tract, such as colorectal, gastric (stomach) and pancreatic cancer, as well as other adenocarcinomas such as breast and lung cancers. CEA is of clinical interest because circulating CEA can be detected in the great majority of patients with CEA-positive tumors. In lung cancer, about 50% of total cases have circulating CEA, with high concentrations of CEA (greater than 20 ng/ml) often detected in adenocarcinomas. Approximately 50% of patients with gastric carcinoma are serologically positive for CEA.

20 The 5'-flanking sequence of the *CEA* gene has been shown to confer cell-specific activity. The *CEA* promoter region, approximately the first 424 nucleotides upstream of the transcriptional start site in the 5' flanking region of the gene, was shown to confer cell-specific activity by virtue of providing higher promoter activity in CEA-producing cells than in non-producing HeLa cells. Schrewe *et al.* (1990) *Mol. Cell. Biol.* 10:2738-2748. In 25 addition, cell-specific enhancer regions have been found. See PCT/GB/02546. The *CEA* promoter, putative silencer, and enhancer elements appears to be contained within a region that extends approximately 14.5 kb upstream from the transcription start site. Richards *et al.* (1995); PCT/GB/02546. Further characterization of the 5'-flanking region of the *CEA* gene by Richards *et al.* (1995) *supra* indicated that two upstream regions (one 30 between about -13.6 and about -10.7 kb, and the other between about -6.1 and about -4.0 kb), when linked to the multimerized promoter, resulted in high-level and selective expression of a reporter construct in CEA-producing LoVo and SW1463 cells. Richards *et al.* (1995) *supra* also localized the promoter region between about nt -90 and

about nt +69 relative to the transcriptional start site, with the region between about nt -41 and about nt -18 being essential for expression. PCT/GB/02546 describes a series of 5'-flanking *CEA* fragments which confer cell-specific activity, including fragments comprising the following sequences: about nt -299 to about nt +69; about nt -90 to about nt +69; nt -14,500 to nt -10,600; nt -13,600 to nt -10,600; and nt -6100 to nt -3800, with all coordinates being relative to the transcriptional start point. In addition, cell-specific transcription activity is conferred on an operably linked gene by the *CEA* fragment from nt -402 to nt +69.

CEA-TREs for use in the vectors disclosed herein are derived from mammalian cells, including, but not limited to, human cells. Thus, any of the *CEA*-TREs can be used as long as the requisite desired functionality is displayed by the vector.

#### Mucin

The protein product of the *MUC1* gene (known as mucin, MUC1 protein; episialin; polymorphic epithelial mucin or PEM; EMA; DF3 antigen; NPGP; PAS-O; or CA15.3 antigen) is normally expressed mainly at the apical surface of epithelial cells lining the glands or ducts of the stomach, pancreas, lungs, trachea, kidney, uterus, salivary glands, and mammary glands. Zotter *et al.* (1988) *Cancer Rev.* 11-12:55-101; and Girling *et al.* (1989) *Int. J. Cancer* 43:1072-1076. However, mucin is overexpressed in 75-90% of human breast carcinomas. Kufe *et al.* (1984) *Hybridoma* 3:223-232. For reviews, see Hilkens (1988) *Cancer Rev.* 11-12:25-54; and Taylor-Papadimitriou, *et al.* (1990) *J. Nucl. Med. Allied Sci.* 34:144-150. Mucin protein expression correlates with the degree of breast tumor differentiation. Lundy *et al.* (1985) *Breast Cancer Res. Treat.* 5:269-276.

Overexpression of the *MUC1* gene in human breast carcinoma cells MCF-7 and ZR-75-1 appears to occur at the transcriptional level. Kufe *et al.* (1984) *supra*; Kovarik (1993) *J. Biol. Chem.* 268:9917-9926; and Abe *et al.* (1990) *J. Cell. Physiol.* 143:226-231. The regulatory sequences of the *MUC1* gene have been cloned, including the approximately 0.9 kb upstream of the transcription start site which contains a TRE that appears to be involved in cell-specific transcription. Abe *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:282-286; Kovarik *et al.* (1993) *supra*; and Kovarik *et al.* (1996) *J. Biol. Chem.* 271:18140-18147.

*MUC1*-TREs are derived from mammalian cells, including but not limited to, human cells. Preferably, the *MUC1*-TRE is human. In one embodiment, the *MUC1*-TRE contains the entire 0.9 kb 5' flanking sequence of the *MUC1* gene. In other embodiments,

*MUC1*-TREs comprise the following sequences (relative to the transcription start site of the *MUC1* gene) operably-linked to a promoter: about nt -725 to about nt +31, about nt -743 to about nt +33, about nt -750 to about nt +33, and about nt -598 to about nt +485.

c-erbB2/HER-2/neu

5 The *c-erbB2/neu* gene (*HER-2/neu* or *HER*) is a transforming gene that encodes a 185 kD epidermal growth factor receptor-related transmembrane glycoprotein. In humans, the *c-erbB2/neu* protein is expressed during fetal development and, in adults, the protein is weakly detectable (by immunohistochemistry) in the epithelium of many normal tissues. Amplification and/or over-expression of the *c-erbB2/neu* gene has been associated with 10 many human cancers, including breast, ovarian, uterine, prostate, stomach and lung cancers. The clinical consequences of overexpression of the *c-erbB2/neu* protein have been best studied in breast and ovarian cancer. *c-erbB2/neu* protein over-expression occurs in 20 to 40% of intraductal carcinomas of the breast and 30% of ovarian cancers, and is associated with a poor prognosis in subcategories of both diseases.

15 Human, rat and mouse *c-erbB2/neu* TREs have been identified and shown to confer transcriptional activity specific to *c-erbB2/neu*-expressing cells. Tal *et al.* (1987) *Mol. Cell. Biol.* 7:2597-2601; Hudson *et al.* (1990) *J. Biol. Chem.* 265:4389-4393; Grooteclaes *et al.* (1994) *Cancer Res.* 54:4193-4199; Ishii *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:4374-4378; and Scott *et al.* (1994) *J. Biol. Chem.* 269:19848-19858.

20 Melanocyte-specific TRE

It has been shown that some genes which encode melanoma proteins are frequently expressed in melanoma/melanocytes, but silent in the majority of normal tissues. A variety of melanocyte-specific TRE are known, are responsive to cellular proteins (transcription factors and/or co-factor(s)) associated with melanocytes, and comprise at least a portion of 25 a melanocyte-specific promoter and/or a melanocyte-specific enhancer. Known transcription factors that control expression of one or more melanocyte-specific genes include the microphthalmia associated transcription factor MITF. Yasumoto *et al.* (1997) *J. Biol. Chem.* 272:503-509. Other transcription factors that control expression of one or more melanocyte specific genes include MART-1/Melan-A, gp100, TRP-1 and TRP-2.

30 Methods are described herein for measuring the activity of a melanocyte-specific TRE and thus for determining whether a given cell allows a melanocyte-specific TRE to function.

In some embodiments, the melanocyte-specific TREs used in this invention are derived from mammalian cells, including but not limited to, human, rat, and mouse. Any melanocyte-specific TREs may be used in the adenoviral vectors of the invention. Rodent and human 5' flanking sequences from genes expressed specifically or preferentially in melanoma cells have been described in the literature and are thus made available for practice of this invention and need not be described in detail herein. The following are some examples of melanocyte-specific TREs which can be used. A promoter and other control elements in the human tyrosinase gene 5' flanking region have been described and sequences have been deposited as GenBank Accession Nos. X16073 and D10751. Kikuchi et al. (1989) *Biochim. Biophys. Acta* 1009:283-286; and Shibata et al. (1992) *J. Biol. Chem.* 267:20584-20588. A cis-acting element has been defined that enhances melanocyte-specific expression of human tyrosinase gene. This element comprises a 20-bp sequence known as tyrosinase distal element (TDE), contains a CATGTG motif, and lies at positions about -1874 to about -1835 relative to the human tyrosinase gene transcription start site.

Yasumoto et al. (1994) *Mol. Cell. Biol.* 14:8058-8070. A promoter region comprising sequences from about -209 to +61 of the human tyrosinase gene was found to direct melanocyte-specific expression. Shibata (1992). Similarly, the mouse tyrosinase 5' flanking region has been analyzed and a sequence deposited as GenBank Accession Nos. D00439 and X51743. Klüppel et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:3777-3788. A minimal promoter has been identified for the mouse TRP-1 gene, and was reported to encompass nucleotides -44 to +107 relative to the transcription start site. Lowings et al. (1992) *Mol. Cell. Biol.* 12:3653-3662. Two regulatory regions required for melanocyte-specific expression of the human TRP-2 gene have been identified. Yokoyama et al. (1994) *J. Biol. Chem.* 269:27080-27087. A human MART-1 promoter region has been described and deposited as GenBank Accession No. U55231. Melanocyte-specific promoter activity was found in a 233-bp fragment of the human MART-1 gene 5' flanking region. Butterfield et al. (1997) *Gene* 191:129-134. A basic-helix-loop-helix/leucine zipper-containing transcription factor, MITF (microphthalmia associated transcription factor) was reported to be involved in transcriptional activation of tyrosinase and TRP-1 genes. Yasumoto et al. (1997) *J. Biol. Chem.* 272:503-509.

In some embodiments, a melanocyte-specific TRE comprises sequences derived from the 5' flanking region of a human tyrosinase gene depicted in Table 3. In some of these embodiments, the melanocyte-specific TRE comprises tyrosinase nucleotides from

about -231 to about +65 relative to the transcription start site (from about nucleotide 244 to about nucleotide 546 of SEQ ID NO:10) and may further comprise nucleotides from about -1956 to about -1716 relative to the human tyrosinase transcription start site (from about nucleotide 6 to about nucleotide 243 of SEQ ID NO:10). A melanocyte-specific TRE can 5 comprise nucleotides from about ---231 to about + 65 juxtaposed to nucleotides from about -1956 to about -1716. It has been reported that nucleotides from about -1956 to about - 1716 relative to the human tyrosinase transcription start site can confer melanocyte-specific expression of an operably linked reporter gene with either a homologous or a heterologous promoter. Accordingly, in some embodiments, a melanocyte-specific TRE comprises 10 nucleotides from about -1956 to about -1716 operably linked to a heterologous promoter.

A melanocyte-specific TRE can also comprise multimers. For example, a melanocyte-specific TRE can comprise a tandem series of at least two, at least three, at least four, or at least five tyrosinase promoter fragments. Alternatively, a melanocyte-specific TRE could have one or more tyrosinase promoter regions along with one or more 15 tyrosinase enhancer regions. These multimers may also contain heterologous promoter and/or enhancer sequences.

#### Cell status-specific TREs

Cell status-specific TREs for use in the adenoviral vectors of the present invention can be derived from any species, preferably a mammal. A number of genes have been 20 described which are expressed in response to, or in association with, a cell status. Any of these cell status-associated genes may be used to generate a cell status-specific TRE.

An example of a cell status is cell cycle. An exemplary gene whose expression is associated with cell cycle is E2F-1, a ubiquitously expressed, growth-regulated gene, which exhibits peak transcriptional activity in S phase. Johnson et al. (1994) *Genes Dev.* 8:1514- 25 1525. The RB protein, as well as other members of the RB family, form specific complexes with E2F-1, thereby inhibiting its ability to activate transcription. Thus, E2F-1-responsive promoters are down-regulated by RB. Many tumor cells have disrupted RB function, which can lead to de-repression of E2F-1-responsive promoters, and, in turn, de-regulated cell division.

30 Accordingly, in one embodiment, the invention provides an E3-containing adenoviral vector in which an adenoviral gene (preferably a gene necessary for replication) is under transcriptional control of a cell status-specific TRE, wherein the cell status-specific

TRE comprises a cell cycle-activated TRE. In one embodiment, the cell cycle-activated TRE is an E2F1 TRE.

Another group of genes that are regulated by cell status are those whose expression is increased in response to hypoxic conditions. Bunn and Poyton (1996) *Physiol. Rev.* 76:839-885; Dachs and Stratford (1996) *Br. J. Cancer* 74:5126-5132; Guillemin and Krasnow (1997) *Cell* 89:9-12. Many tumors have insufficient blood supply, due in part to the fact that tumor cells typically grow faster than the endothelial cells that make up the blood vessels, resulting in areas of hypoxia in the tumor. Folkman (1989) *J. Natl. Cancer Inst.* 82:4-6; and Kallinowski (1996) *The Cancer J.* 9:37-40. An important mediator of hypoxic responses is the transcriptional complex HIF-1, or hypoxia inducible factor-1, which interacts with a hypoxia-responsive element (HRE) in the regulatory regions of several genes, including vascular endothelial growth factor, and several genes encoding glycolytic enzymes, including enolase-1. Murine HRE sequences have been identified and characterized. Firth et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:6496-6500. An HRE from a rat enolase-1 promoter is described in Jiang et al. (1997) *Cancer Res.* 57:5328-5335. An HRE from a rat enolase-1 promoter is depicted in Table 3.

Accordingly, in one embodiment, an adenovirus vector comprises an adenovirus gene, preferably an adenoviral gene essential for replication, under transcriptional control of a cell status-specific TRE comprising an HRE. In one embodiment, the cell status-specific TRE comprises the HRE depicted in Table 3.

Other cell status-specific TREs include heat-inducible (i.e., heat shock) promoters, and promoters responsive to radiation exposure, including ionizing radiation and UV radiation. For example, the promoter region of the early growth response-1 (Egr-1) gene contains an element(s) inducible by ionizing radiation. Hallahan et al. (1995) *Nat. Med.* 1:786-791; and Tsai-Morris et al. (1988) *Nucl. Acids. Res.* 16:8835-8846. Heat-inducible promoters, including heat-inducible elements, have been described. See, for example Welsh (1990) in "Stress Proteins in Biology and Medicine", Morimoto, Tissieres, and Georgopoulos, eds. Cold Spring Harbor Laboratory Press; and Perisic et al. (1989) *Cell* 59:797-806. Accordingly, in some embodiments, the cell status-specific TRE comprises an element(s) responsive to ionizing radiation. In one embodiment, this TRE comprises a 5' flanking sequence of an Egr-1 gene. In other embodiments, the cell status-specific TRE comprises a heat shock responsive element.

The cell status-specific TREs listed above are provided as non-limiting examples of TREs that would function in the instant invention. Additional cell status-specific TREs are known in the art, as are methods to identify and test cell status specificity of suspected cell status-specific TREs.

5           *Urothelial cell-specific TREs*

Any urothelial cell-specific TRE may be used in the adenoviral vectors of the invention. A number of urothelial cell-specific proteins have been described, among which are the uroplakins. Uroplakins (UP), including UPIa and UPIb (27 and 28 kDa, respectively), UPII (15 kDa), and UPIII (47 kDa), are members of a group of integral membrane proteins that are major proteins of urothelial plaques. These plaques cover a large portion of the apical surface of mammalian urothelium and may play a role as a permeability barrier and/or as a physical stabilizer of the urothelial apical surface. Wu et al. (1994) *J. Biol. Chem.* 269:13716-13724. UPs are bladder-specific proteins, and are expressed on a significant proportion of urothelial-derived tumors, including about 88% of transitional cell carcinomas. Moll et al. (1995) *Am. J. Pathol.* 147:1383-1397; and Wu et al. (1998) *Cancer Res.* 58:1291-1297. The control of the expression of the human UPII has been studied, and a 3.6-kb region upstream of the mouse UPII gene has been identified which can confer urothelial-specific transcription on heterologous genes (Lin et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:679-683).

20           Preferred urothelial cell-specific TREs include TREs derived from the uroplakins UPIa, UPIb, UPII, and UPIII, as well as urohingin. A uroplakin TRE may be from any species, depending on the intended use of the adenovirus, as well as the requisite functionality is exhibited in the target or host cell. Significantly, adenovirus constructs comprising a urothelial cell-specific TREs have observed that such constructs are capable 25 of selectively replicating in urothelial cells as opposed to smooth muscle cells, which adjoin urothelial cells in the bladder.

*Uroplakin*

Urothelial-specific TREs derived from the *hUPII* gene are described herein. Accordingly, in some embodiments, an adenovirus vector of the invention comprises an 30 adenovirus gene, preferably an adenoviral gene essential for replication, under transcriptional control of a urothelial cell-specific TRE which comprises the 2.2 kb sequence from the 5' flanking region of *hUPII* gene, as shown in Table 3. In other embodiments, an adenovirus vector of the invention comprises an adenovirus gene,

preferably an adenoviral gene essential for replication, under transcriptional control of a urothelial cell-specific TRE which comprises a 1.8 kb sequence from the 5' flanking region of hUPII gene, from nucleotides 430 to 2239 as shown in Table 3. In other embodiments, the urothelial cell-specific TRE comprises a functional portion of the 2.2 kb sequence depicted in Table 3, or a functional portion of the 1.8 kb sequence of nucleotides 430 to 2239 of the sequence depicted in Table 3, such as a fragment of 2000 bp or less, 1500 bp or less, or 1000 bp or less, 600 bp less, or at least 200 bp which includes the 200 bp fragment of the hUPII 5'-flanking region.

5 A 3.6 kb 5'-flanking sequence located from the mouse UPII (mUPII) gene which 10 confers urothelial cell-specific transcription on heterologous genes is one urothelial cell-specific TRE useful in vectors of the instant invention (Table 3). Smaller TREs (*i.e.*, 3500 bp or less, more preferably less than about 2000 bp, 1500 bp, or 1000 bp) are preferred. Smaller TREs derived from the mUPII 3.6 kb fragment are one group of preferred 15 urothelial cell-specific TREs. In particular, Inventors have identified an approximately 600 bp fragment from the 5' flanking DNA of the mUPII gene, which contains 540 bp of 5' untranslated region (UTR) of the mUPII gene, that confers urothelial cell-specific expression on heterologous genes.

Accordingly, in some embodiments, an adenovirus vector of the invention 20 comprises an adenovirus gene, preferably an adenoviral gene essential for replication, under transcriptional control of a urothelial cell-specific TRE which comprises the 3.6 kb sequence from the 5' flanking region of mouse UPII gene, as shown in Table 3. In other embodiments, the urothelial cell-specific TRE comprises a functional portion of the 3.6 kb sequence depicted in Table 3, such as a fragment of 3500 bp or less, 2000 bp or less, 1500 bp or less, or 1000 bp or less which includes the 540 bp fragment of 5' UTR. The 25 urothelial cell-specific TRE may also be a sequence which is substantially identical to the 3.6 kb mUPII 5'-flanking region or any of the described fragments thereof.

As an example of how urothelial cell-specific TRE activity can be determined, a 30 polynucleotide sequence or set of such sequences can be generated using methods known in the art, such as chemical synthesis, site-directed mutagenesis, PCR, and/or recombinant methods. The sequence(s) to be tested is inserted into a vector containing an appropriate reporter gene, including, but not limited to, chloramphenicol acetyl transferase (CAT),  $\beta$ -galactosidase (encoded by the lacZ gene), luciferase (encoded by the luc gene), a green fluorescent protein, alkaline phosphatase, and horse radish peroxidase. Such vectors and

assays are readily available, from, *inter alia*, commercial sources. Plasmids thus constructed are transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative target cell-specific TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes (lipofection) and DEAE dextran. Suitable host cells include any urothelial cell type, including but not limited to, KU-1, MYP3 (a non-tumorigenic rat urothelial cell line), 804G (rat bladder carcinoma cell line), cultured human urothelial cells (HUC), HCV-29, UM-UC-3, SW780, RT4, HL60, KG-1, and KG-1A. Non-urothelial cells, such as LNCaP, HBL-100, HLF, HLE, 3T3, Hep3B, HuH7, CADO-LC9, and HeLa are used as a control.

10 Results are obtained by measuring the level of expression of the reporter gene using standard assays. Comparison of expression between urothelial cells and control indicates presence or absence of transcriptional activation.

Comparisons between or among various urothelial cell-specific TREs can be assessed by measuring and comparing levels of expression within a single urothelial cell line. It is understood that absolute transcriptional activity of a urothelial cell-specific TRE will depend on several factors, such as the nature of the target cell, delivery mode and form of the urothelial cell-specific TRE, and the coding sequence that is to be selectively transcriptionally activated. To compensate for various plasmid sizes used, activities can be expressed as relative activity per mole of transfected plasmid. Alternatively, the level of transcription (i.e., mRNA) can be measured using standard Northern analysis and hybridization techniques. Levels of transfection (i.e., transfection efficiencies) are measured by co-transfected a plasmid encoding a different reporter gene under control of a different TRE, such as the CMV immediate early promoter. This analysis can also indicate negative regulatory regions, i.e., silencers.

25 Alternatively a putative urothelial cell-specific TRE can be assessed for its ability to confer adenoviral replication preference for cells that allow a urothelial cell-specific TRE to function. For this assay, constructs containing an adenovirus gene essential to replication operatively linked to a putative urothelial cell-specific TRE are transfected into urothelial cells. Viral replication in those cells is compared, for example, to viral replication by wild type adenovirus in those cells and/or viral replication by the construct in non-urothelial cells.

*TRE configurations*

A TRE as used in the present invention can be present in a variety of configurations. A TRE can comprise multimers. For example, a TRE can comprise a tandem series of at least two, at least three, at least four, or at least five target cell-specific TREs. These 5 multimers may also contain heterologous promoter and/or enhancer sequences.

2 Optionally, a transcriptional terminator or transcriptional "silencer" can be placed upstream of the target cell-specific TRE, thus preventing unwanted read-through transcription of the coding segment under transcriptional control of the target cell-specific TRE. Also, optionally, the endogenous promoter of the coding segment to be placed under 10 transcriptional control of the target cell-specific TRE can be deleted.

A target cell-specific TRE may or may not lack a silencer. The presence of a silencer (i.e., a negative regulatory element) may assist in shutting off transcription (and thus replication) in non-permissive cells (i.e., a non-target cell). Thus, presence of a silencer may confer enhanced target cell-specific replication by more effectively preventing 15 adenoviral vector replication in non-target cells. Alternatively, lack of a silencer may assist in effecting replication in target cells, thus conferring enhanced target cell-specific replication due to more effective replication in target cells.

It is also understood that the invention includes a target cell-specific TRE regulating the transcription of a bicistronic mRNA in which translation of the second mRNA is 20 associated by an IRES. An adenovirus vector may further include an additional heterologous TRE which may or may not be operably linked to the same gene(s) as the target cell-specific TRE. For example a TRE (such as a cell type-specific or cell status-specific TRE) may be juxtaposed to a second type of target-cell-specific TRE.

25 "Juxtaposed" means a target cell-specific TRE and a second TRE transcriptionally control the same gene. For these embodiments, the target cell-specific TRE and the second TRE may be in any of a number of configurations, including, but not limited to, (a) next to each other (i.e., abutting); (b) both 5' to the gene that is transcriptionally controlled (i.e., may have intervening sequences between them); (c) one TRE 5' and the other TRE 3' to the gene.

30 As is readily appreciated by one skilled in the art, a target cell-specific TRE is a polynucleotide sequence, and, as such, can exhibit function over a variety of sequence permutations. Methods of nucleotide substitution, addition, and deletion are known in the art, and readily available functional assays (such as the CAT or luciferase reporter gene

assay) allow one of ordinary skill to determine whether a sequence variant exhibits requisite target cell-specific transcription function. Hence, the invention also includes functionally-preserved variants of the TRE nucleic acid sequences disclosed herein, which include nucleic acid substitutions, additions, and/or deletions. The variants of the 5 sequences disclosed herein may be 80%, 85%, 90%, 95%, 98%, 99% or more identical, as measured by, for example, ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using default parameters, which are as follows: mismatch = 2; open gap = 0; extend gap = 2 to any of the urothelial cell-specific TRE sequences disclosed herein. Variants of target cell-specific TRE sequences may also hybridize at high 10 stringency, that is at 68°C and 0.1X SSC, to any of the target cell-specific TRE sequences disclosed herein.

In terms of hybridization conditions, the higher the sequence identity required, the more stringent are the hybridization conditions if such sequences are determined by their ability to hybridize to a sequence of TRE disclosed herein. Accordingly, the invention also 15 includes polynucleotides that are able to hybridize to a sequence comprising at least about 15 contiguous nucleotides (or more, such as about 25, 35, 50, 75 or 100 contiguous nucleotides) of a TRE disclosed herein. The hybridization conditions would be stringent, i.e., 80°C (or higher temperature) and 6M SSC (or less concentrated SSC). Another set of stringent hybridization conditions is 68°C and 0.1 X SSC. For discussion regarding 20 hybridization reactions, see below.

Hybridization reactions can be performed under conditions of different “stringency”. Conditions that increase stringency of a hybridization reaction of widely known and published in the art. See, for example, Sambrook et al. (1989) at page 7.52. Examples of relevant conditions include (in order of increasing stringency): incubation 25 temperatures of 25°C, 37°C, 50°C and 68°C; buffer concentrations of 10 X SSC, 6 X SSC, 1 X SSC, 0.1 X SSC (where SSC is 0.15 M NaCl and 15 mM citrate buffer) and their equivalents using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours; 1, 2, or more washing steps; wash 30 incubation times of 1, 2, or 15 minutes; and wash solutions of 6 X SSC, 1 X SSC, 0.1 X SSC, or deionized water. An exemplary set of stringent hybridization conditions is 68°C and 0.1 X SSC.

“T<sub>m</sub>” is the temperature in degrees Celcius at which 50% of a polynucleotide duplex made of complementary strands hydrogen bonded in anti-parallel direction by Watson-

Crick base pairing dissociates into single strands under conditions of the experiment.  $T_m$  may be predicted according to a standard formula, such as:

$$T_m = 81.5 + 16.6 \log[X^+] + 0.41 (\%G/C) - 0.61 (\%F) - 600/L$$

5

where  $[X^+]$  is the cation concentration (usually sodium ion,  $Na^+$ ) in mol/L; (%G/C) is the number of G and C residues as a percentage of total residues in the duplex; (%F) is the percent formamide in solution (wt/vol); and L is the number of nucleotides in each strand of the duplex.

10 While not wishing to be bound by a single theory, the inventors note that it is possible that certain modifications will result in modulated resultant expression levels, including enhanced expression levels. Achievement of modulated resultant expression levels, preferably enhanced expression levels, may be especially desirable in the case of certain, more aggressive forms of cancer, or when a more rapid and/or aggressive pattern of 15 cell killing is warranted (due to an immunocompromised condition of the individual, for example).

*Determination of TRE activity*

Activity of a TRE can be determined, for example, as follows. A TRE polynucleotide sequence or set of such sequences can be generated using methods known 20 in the art, such as chemical synthesis, site-directed mutagenesis, PCR, and/or recombinant methods. The sequence(s) to be tested can be inserted into a vector containing a promoter (if no promoter element is present in the TRE) and an appropriate reporter gene encoding a reporter protein, including, but not limited to, chloramphenicol acetyl transferase (CAT),  $\beta$ -galactosidase (encoded by the *lacZ* gene), luciferase (encoded by the *luc* gene), alkaline 25 phosphatase (AP), green fluorescent protein (GFP), and horseradish peroxidase (HRP). Such vectors and assays are readily available, from, *inter alia*, commercial sources. Plasmids thus constructed are transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes, DEAE 30 dextran-mediated transfer, particle bombardment or direct injection. TRE activity is measured by detection and/or quantitation of reporter gene-derived mRNA and/or protein. Reporter protein product can be detected directly (e.g., immunochemically) or through its enzymatic activity, if any, using an appropriate substrate. Generally, to determine cell

specific activity of a TRE, a TRE-reporter gene construct is introduced into a variety of cell types. The amount of TRE activity is determined in each cell type and compared to that of a reporter gene construct lacking the TRE. A TRE is determined to be cell-specific if it is preferentially functional in one cell type, compared to a different type of cell.

5        *Adenovirus early genes*

The adenovirus vectors of the invention comprise two or more genes which are co-transcribed under the control of a target cell-specific TRE wherein the second gene is under translational control of an IRES. One or more of the genes can be an adenovirus gene, preferably an adenovirus gene essential for replication. Any gene that is essential for 10 adenovirus replication, such as E1A, E1B, E2, E4 or any of the late genes, is useful. The adenovirus may also comprise E3. In addition, one or more of the genes can be a transgene or heterologous gene. Any of the various adenovirus serotypes can be used, such as, for example, Ad2, Ad5, Ad12 and Ad40. For purposes of illustration, the Ad5 serotype is exemplified herein.

15        The E1A gene is expressed immediately (between 0 and 2 hours) after viral infection, before any other viral genes. E1A protein is a *trans*-acting positive transcriptional regulatory factor, and is required for the expression of the other early viral genes E1B, E2, E3, E4, and the promoter-proximal major late genes. Despite the nomenclature, the promoter proximal genes driven by the major late promoter are also 20 expressed during early times after Ad5 infection. Flint (1982) *Biochem. Biophys. Acta* **651**:175-208; Flint (1986) *Advances Virus Research* **31**:169-228; and Grand (1987) *Biochem. J.* **241**:25-38. In the absence of a functional E1A gene, viral infection does not proceed, because the gene products necessary for viral DNA replication are not produced. Nevins (1989) *Adv. Virus Res.* **31**:35-81. The transcription start site of Ad5 E1A is at 25 coordinate 498 and the ATG start site of the E1A protein is at coordinate 560 in the virus genome.

The E1B protein is necessary in *trans* for transport of late mRNA from the nucleus to the cytoplasm. Defects in E1B expression result in poor expression of late viral proteins and an inability to shut off host cell protein synthesis. The promoter of E1B has been 30 implicated as the defining element of difference in the host range of Ad40 and Ad5: clinically Ad40 is an enterovirus, whereas Ad5 causes acute conjunctivitis. Bailey *et al.* (1993) *Virology* **193**:631; Bailey *et al.* (1994) *Virology* **202**:695-706. The E1B promoter

of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box, and extends from Ad5 nt 1636 to 1701.

Adenovirus E1B 19-kDa (19K) protein is a potent inhibitor of apoptosis and cooperates with E1A to produce oncogenic transformation of primary cells (Rao, et al., 5 1992, *Cell Biology*, 89:7742-7746). During productive adenovirus infection, E1A stimulates host cell DNA synthesis, thereby causing cells to aberrantly go through the cell cycle. In response to cell cycle deregulation, the host cell undergoes apoptosis. As a defense mechanism, the E1B 19-kDa protein inhibits this E1A-induced apoptosis and allows assembly of viral progeny to be completed before the cell commits suicide. E1B 19- 10 kDa conducts anti-apoptotic function by multiple mechanisms. E1B 19-kDa inhibits the apoptosis of multiple stimuli, including E1a, p53 and TNF, for example. According to wild-type Ad5, the E1B 19-kDa region is located between nucleotide 1714 and nucleotide 2244. The E1B 19-kDa region has been described in, for example, Rao *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:7742-7746.

15 In a preferred embodiment, expression of the E1A and E1B regions of the Ad genome is facilitated in a cell-specific fashion by placing a cell-specific TRE upstream of E1A and a internal ribosome entry site between E1A and E1B.

20 The E2 region of adenovirus encodes proteins related to replication of the adenoviral genome, including the 72 kD DNA-binding protein, the 80 kD precursor terminal protein and the viral DNA polymerase. The E2 region of Ad5 is transcribed in a rightward orientation from two promoters, termed E2 early and E2 late, mapping at 76.0 and 72.0 map units, respectively. While the E2 late promoter is transiently active during late stages of infection and is independent of the E1A transactivator protein, the E2 early promoter is crucial during the early phases of viral replication.

25 The E2 early promoter of Ad5 is located between nucleotides 27,050 and 27,150, and consists of a major and a minor transcription initiation site (the latter accounting for about 5% of E2 transcripts), two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site. For a detailed review of E2 promoter architecture see Swaminathan *et al.* (1995) *Curr. Topics in Micro. and Imm.* 199 30 part 3:177-194.

The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable for genetic manipulation. However, the E2 early promoter overlaps by only a few base pairs with sequences on the counterstrand

which encode a 33 kD protein. Notably, an SpeI restriction site (Ad5 position 27,082) is part of the stop codon for the above mentioned 33 kD protein and conveniently separates the major E2 early transcription initiation site and TATA box from the upstream E2F and ATF binding sites. Therefore, insertion of a heterologous TRE having SpeI ends into the 5 SpeI site disrupts the endogenous E2 early promoter of Ad5 and allows TRE-regulated expression of E2 transcripts.

An E3 region refers to the region of the adenoviral genome that encodes the E3 products. The E3 region has been described in various publications, including, for example, Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199:237-274. Generally, 10 the E3 region is located between about 28583 and about 30470 of the adenoviral genome. An E3 region for use in the present invention may be from any adenovirus serotype. An E3 sequence is a polynucleotide sequence that contains a sequence from an E3 region. In some embodiments, the sequence encodes ADP. In other embodiments, the sequence encodes other than ADP and excludes a sequence encoding only ADP. As is well known in 15 the art, the ADP coding region is located in the E3 region within the adenoviral genome from about 29468 bp to about 29773 bp; including the Y leader, the location of ADP is from about 28375 bp to about 29773 bp for Ad5. Other ADP regions for other serotypes are known in the art. An E3 sequence includes, but is not limited to, deletions; insertions; fusions; and substitutions. An E3 sequence may also comprise an E3 region or a portion of 20 the E3 region. It is understood that, as an "E3 sequence" is not limited to an "E3 region", alternative references herein to an "E3 region" or "E3 sequence" do not indicate that these terms are interchangeable. Assays for determining a functional E3 sequence for purposes of this invention are described herein.

The E4 gene has a number of transcription products and encodes two polypeptides 25 (the products of open reading frames (ORFs) 3 and 6) which are responsible for stimulating the replication of viral genomic DNA and stimulating late gene expression, through interaction with heterodimers of cellular transcription factors E2F-1 and DP-1. The ORF 6 protein requires interaction with the E1B 55 kD protein for activity while the ORF 3 protein does not. In the absence of functional ORF 3- and ORF 6-encoded proteins, efficiency of 30 plaque formation is less than  $10^{-6}$  that of wild type virus.

To further increase cell-specificity of replication, it is possible to take advantage of the interaction between the E4 ORF 6 gene product and the E1B 55 kD protein. For example, if E4 ORFs 1-3 are deleted, viral DNA replication and late gene synthesis

becomes dependent on E4 ORF6 protein. By generating such a deletion in a vector in which the E1B region is regulated by a cell-specific TRE, a virus is obtained in which both E1B and E4 functions are dependent on the cell-specific TRE which regulates E1B.

Late genes relevant to the disclosed vectors are L1, L2 and L3, which encode 5 proteins of the virion. All of these genes (typically coding for structural proteins) are probably required for adenoviral replication. All late genes are under the control of the major late promoter (MLP), which is located in Ad5 between nucleotides 5986 and 6048.

In one embodiment, an adenovirus early gene is under transcriptional control of a cell specific, heterologous TRE. In additional embodiments, the early gene is selected from 10 the group including E1A, E1B, E2, E3, E4. In another embodiment, an adenovirus late gene is under transcriptional control of a cell specific, heterologous TRE. In further embodiments, two or more early genes are under the control of heterologous TREs that function in the same target cell. The heterologous TREs can be the same or different, or one can be a variant of the other. In additional embodiments, two or more late genes are 15 under the control of heterologous TREs that function in the same target cell. The heterologous TREs can be the same or different, or one can be a variant of the other. In yet another embodiment, one or more early gene(s) and one or more late gene(s) are under transcriptional control of the same or different heterologous TREs, wherein the TREs function in the same target cell.

20 In some embodiments of the present invention, the adenovirus vector comprises the essential gene E1A and the E1A promoter is deleted. In other embodiments, the adenovirus vector comprises the essential gene E1A and the E1A enhancer I is deleted. In yet other embodiments, the E1A promoter is deleted and E1A enhancer I is deleted. In other embodiments, an internal ribosome entry site (IRES) is inserted upstream of E1B (so that 25 E1B is translationally linked), and a target cell-specific TRE is operably linked to E1A. In still other embodiments, an (IRES) is inserted upstream of E1B (so that E1B is translationally linked), and target cell-specific TRE is operably linked to E1A, which may or may not maintain the E1A promoter and/or enhancer I (i.e., the E1A promoter and/or enhancer I may be, but not necessarily be, deleted). In yet other embodiments, the 19-kDa 30 region of E1B is deleted.

For adenovirus vectors comprising a second gene under control of an IRES, it is preferred that the endogenous promoter of a gene under translational control of an IRES be deleted so that the endogenous promoter does not interfere with transcription of the second

gene. It is preferred that the second gene be in frame with the IRES if the IRES contains an initiation codon. If an initiation codon, such as ATG, is present in the IRES, it is preferred that the initiation codon of the second gene is removed and that the IRES and second gene are in frame. Alternatively, if the IRES does not contain an initiation codon or if the 5 initiation codon is removed from the IRES, the initiation codon of the second gene is used.

*Adenovirus death protein (ADP) gene and gene product*

In the construction of adenovirus vectors, the E3 region is often deleted to facilitate insertion of one or more TREs and/or transgenes. In some embodiments, however, the adenovirus death protein (ADP), encoded within the E3 region, is retained in an adenovirus 10 vector. The ADP gene, under control of the major late promoter (MLP), appears to code for a protein (ADP) that is important in expediting host cell lysis. Tollefson *et al.* (1992) *J. Virol.* 66:3633; and Tollefson *et al.* (1996) *J. Virol.* 70:2296. Thus, inclusion of an ADP gene in a viral vector can render the vector more potent, making possible more effective treatment and/or a lower dosage requirement.

15 An ADP coding sequence is obtained preferably from Ad2 (since this is the strain in which the ADP has been most fully characterized) using techniques known in the art, such as PCR. Preferably, the Y leader (which is an important sequence for correct expression of late genes) is also obtained and placed in operative linkage to the ADP coding sequence. The ADP coding sequence (with or without the Y leader) is then introduced into an 20 adenoviral genome, for example, in the E3 region, where expression of the ADP coding sequence will be driven by the MLP. The ADP coding sequence can, of course, also be inserted in other locations of the adenovirus genome, such as the E4 region. Alternatively, the ADP coding sequence can be operably linked to a heterologous TRE, including, but not limited to, another viral TRE or a target cell-specific TRE (see *infra*). In another 25 embodiment, the ADP gene is present in a viral genome such that it is transcribed as part of a multi-cistronic mRNA in which its translation is associated with an IRES.

*E3-containing target cell-specific adenoviral vectors*

In some embodiments, the adenovirus vectors contain an E3 region, or a portion of 30 an E3 region. Inclusion of the E3 region of adenovirus can enhance cytotoxicity of the target cell-specific adenoviral vectors of the present invention. Adenoviral vectors containing an E3 region may maintain their high level of specificity and can be (a) significantly more cytotoxic; (b) produce higher virus yield including extracellular virus yield; (c) form larger plaques; (d) produce rapid cell death; and (e) kill tumors more

efficiently *in vivo* than vectors lacking the E3 region. The adenoviral vectors of this invention may contain the E3 region or a portion of the E3 region. It is understood that, as inclusion of E3 confers observable and measurable functionality on the adenoviral vectors, for example, increased replication and production, functionally equivalent (in which 5 functionality is essentially maintained, preserved, or even enhanced or diminished) variants of E3 may be constructed. For example, portions of E3 may be used. A portion may be, non-inclusively, either of the following: (a) deletion, preferably at the 3' end; (b) inclusion of one or more various open reading frames of E3. Five proteins which are encoded by the 10 Ad-E3 region have been identified and characterized: (1) a 19-kDa glycoprotein (gpl9k) is one of the most abundant adenovirus early proteins, and is known to inhibit transport of the major histocompatibility complex class I molecules to the cell surface, thus impairing both peptide recognition and clearance of Ad-infected cells by cytotoxic T lymphocytes (CTLs); (2) E3 14.7k protein and the E3 10.4k/14.5k complex of proteins inhibit the cytotoxic and 15 inflammatory responses mediated by tumor necrosis factor (TNF); (3) E3 10.4k/14.5k protein complex down regulates the epidermal growth factor receptor, which may inhibit inflammation and activate quiescent infected cells for efficient virus replication; (4) E3 11.6k protein (adenoviral death protein, ADP) from adenovirus 2 and 5 appears to promote cell death and release of virus from infected cells. The functions of three E3-encoded 20 proteins -- 3.6k, 6.7k and 12.5k -- are unknown. A ninth protein having a molecular weight of 7.5 kDa has been postulated to exist, but has not been detected in cells infected with wild-type adenovirus. Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199:237-274. The E3 region is schematically depicted in FIG. 6. These intact, portions, or variants of E3 25 may be readily constructed using standard knowledge and techniques in the art. Preferably, an intact E3 region is used.

25 In the adenovirus vectors of the present invention, E3 may or may not be under transcriptional control of native adenoviral transcriptional control element(s). The E3 promoter is located within the coding sequence for virion protein VIII, an essential protein which is highly conserved among adenovirus serotypes. In some embodiments, E3 is under transcriptional control of a heterologous TRE, including, but not limited to, a target cell-specific TRE. Accordingly, in one embodiment, the invention provides an adenoviral 30 vector, preferably replication competent, that comprises E3 region (or a portion of E3) under transcriptional control of a target cell-specific TRE. In other embodiments, the E3 region is under transcriptional control of a native adenoviral TRE, and the vector further

comprises an adenoviral gene essential for replication under transcriptional control of a target cell-specific TRE. In other embodiments, the E3 region is under transcriptional control of a target cell-specific TRE, and the vector further comprises an adenoviral gene essential for replication under transcriptional control of a target cell-specific TRE.

5        *Transgenes under transcriptional control of a target cell-specific TRE*

Various other replication-competent adenovirus vectors can be made according to the present invention in which, in addition to having a single or multiple adenovirus gene(s) under control of a target cell-specific TRE, a transgene(s) is/are also under control of a target cell-specific TRE and optionally under translational control of an IRES. Transgenes 10 include, but are not limited to, therapeutic transgenes and reporter genes.

Reporter genes

For example, a target cell-specific TRE can be introduced into an adenovirus vector immediately upstream of and operably linked to an early gene such as E1A or E1B, and this construct may further comprise a second co-transcribed gene under translational 15 control of an IRES. The second gene may be a reporter gene. The reporter gene can encode a reporter protein, including, but not limited to, chloramphenicol acetyl transferase (CAT),  $\beta$ -galactosidase (encoded by the *lacZ* gene), luciferase, alkaline phosphatase, a green fluorescent protein, and horse radish peroxidase. For detection of a putative cancer cell(s) in a biological sample, the biological sample may be treated with modified 20 adenoviruses in which a reporter gene (e.g., luciferase) is under control of a target cell-specific TRE. The target cell-specific TRE will be transcriptionally active in cells that allow the target cell-specific TRE to function, and luciferase will be produced. This production will allow detection of target cells, including cancer cells in, for example, a human host or a biological sample. Alternatively, an adenovirus can be constructed in 25 which a gene encoding a product conditionally required for survival (e.g., an antibiotic resistance marker) is under transcriptional control of a target cell-specific TRE. When this adenovirus is introduced into a biological sample, the target cells will become antibiotic resistant. An antibiotic can then be introduced into the medium to kill the non-cancerous cells.

30        Therapeutic transgenes

Transgenes also include genes which may confer a therapeutic effect, such as enhancing cytotoxicity so as to eliminate unwanted target cells. In this way, various genetic capabilities may be introduced into target cells, particularly cancer cells. For

example, in certain instances, it may be desirable to enhance the degree and/or rate of cytotoxic activity, due to, for example, the relatively refractory nature or particular aggressiveness of the cancerous target cell. This could be accomplished by coupling the target cell-specific cytotoxic activity with cell-specific expression of, for example, HSV-tk and/or cytosine deaminase (cd), which renders cells capable of metabolizing 5-fluorocytosine (5-FC) to the chemotherapeutic agent 5-fluorouracil (5-FU). Using these types of transgenes may also confer a bystander effect.

Other desirable transgenes that may be introduced via an adenovirus vector(s) include genes encoding cytotoxic proteins, such as the A chains of diphtheria toxin, ricin or abrin (Palmiter et al. (1987) *Cell* 50: 435; Maxwell et al. (1987) *Mol. Cell. Biol.* 7: 1576; Behringer et al. (1988) *Genes Dev.* 2: 453; Messing et al. (1992) *Neuron* 8: 507; Piatak et al. (1988) *J. Biol. Chem.* 263: 4937; Lamb et al. (1985) *Eur. J. Biochem.* 148: 265; Frankel et al. (1989) *Mol. Cell. Biol.* 9: 415), genes encoding a factor capable of initiating apoptosis, sequences encoding antisense transcripts or ribozymes, which among other capabilities may be directed to mRNAs encoding proteins essential for proliferation, such as structural proteins, or transcription factors; viral or other pathogenic proteins, where the pathogen proliferates intracellularly; genes that encode an engineered cytoplasmic variant of a nuclease (e.g. RNase A) or protease (e.g. awsin, papain, proteinase K, carboxypeptidase, etc.), or encode the Fas gene, and the like. Other genes of interest include cytokines, antigens, transmembrane proteins, and the like, such as IL-1, -2, -6, -12, GM-CSF, G-CSF, M-CSF, IFN- $\alpha$ , - $\beta$ , - $\gamma$ , TNF- $\alpha$ , - $\beta$ , TGF- $\alpha$ , - $\beta$ , NGF, and the like. The positive effector genes could be used in an earlier phase, followed by cytotoxic activity due to replication.

#### *Host cells*

The present invention also provides host cells comprising (i.e., transformed with) the adenoviral vectors described herein. Both prokaryotic and eukaryotic host cells can be used as long as sequences requisite for maintenance in that host, such as appropriate replication origin(s), are present. For convenience, selectable markers are also provided. Host systems are known in the art and need not be described in detail herein. Prokaryotic host cells include bacterial cells, for example, *E. coli*, *B. subtilis*, and mycobacteria. Among eukaryotic host cells are yeast, insect, avian, plant, *C. elegans* (or nematode) and mammalian host cells. Examples of fungi (including yeast) host cells are *S. cerevisiae*, *Kluyveromyces lactis* (*K. lactis*), species of *Candida* including *C. albicans* and *C. glabrata*,

*Aspergillus nidulans*, *Schizosaccharomyces pombe* (*S. pombe*), *Pichia pastoris*, and *Yarrowia lipolytica*. Examples of mammalian cells are cultured human target cells (HUC), KU-1, MYP3 (a non-tumorigenic rat target cell line), 804G (rat bladder carcinoma cell line), HCV-29, UM-UC-3, SW780, RT4, HL60, KG-1, and KG-1A. COS cells, mouse L cells, LNCaP cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, and African green monkey cells. *Xenopus laevis* oocytes, or other cells of amphibian origin, may also be used.

*Compositions and kits*

The present invention also includes compositions, including pharmaceutical compositions, containing the adenoviral vectors described herein. Such compositions are useful for administration *in vivo*, for example, when measuring the degree of transduction and/or effectiveness of cell killing in an individual. Compositions can comprise an adenoviral vector(s) of the invention and a suitable solvent, such as a physiologically acceptable buffer. These are well known in the art. In other embodiments, these compositions further comprise a pharmaceutically acceptable excipient. These compositions, which can comprise an effective amount of an adenoviral vector of this invention in a pharmaceutically acceptable excipient, are suitable for systemic or local administration to individuals in unit dosage forms, sterile parenteral solutions or suspensions, sterile non-parenteral solutions or oral solutions or suspensions, oil in water or water in oil emulsions and the like. Formulations for parenteral and nonparenteral drug delivery are known in the art and are set forth in *Remington's Pharmaceutical Sciences*, 19th Edition, Mack Publishing (1995). Compositions also include lyophilized and/or reconstituted forms of the adenoviral vectors (including those packaged as a virus, such as adenovirus) of the invention.

The present invention also encompasses kits containing an adenoviral vector(s) of this invention. These kits can be used for diagnostic and/or monitoring purposes, preferably monitoring. Procedures using these kits can be performed by clinical laboratories, experimental laboratories, medical practitioners, or private individuals. Kits embodied by this invention allow someone to detect the presence of bladder cancer cells in a suitable biological sample, such as biopsy specimens.

The kits of the invention comprise an adenoviral vector described herein in suitable packaging. The kit may optionally provide additional components that are useful in the

procedure, including, but not limited to, buffers, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information.

*Preparation of the adenovirus vectors of the invention*

The adenovirus vectors of this invention can be prepared using recombinant techniques that are standard in the art. Generally, a target cell-specific TRE is inserted 5' to the adenoviral gene of interest, preferably an adenoviral replication gene, more preferably one or more early replication genes (although late gene(s) can be used). A target cell-specific TRE can be prepared using oligonucleotide synthesis (if the sequence is known) or recombinant methods (such as PCR and/or restriction enzymes). Convenient restriction sites, either in the natural adeno-DNA sequence or introduced by methods such as PCR or site-directed mutagenesis, provide an insertion site for a target cell-specific TRE. Accordingly, convenient restriction sites for annealing (i.e., inserting) a target cell-specific TRE can be engineered onto the 5' and 3' ends of a UP-TRE using standard recombinant methods, such as PCR.

Polynucleotides used for making adenoviral vectors of this invention may be obtained using standard methods in the art, such as chemical synthesis, recombinant methods and/or obtained from biological sources.

Adenoviral vectors containing all replication-essential elements, with the desired elements (e.g., E1A) under control of a target cell-specific TRE, are conveniently prepared by homologous recombination or *in vitro* ligation of two plasmids, one providing the left-hand portion of adenovirus and the other plasmid providing the right-hand region, one or more of which contains at least one adenovirus gene under control of a target cell-specific TRE. If homologous recombination is used, the two plasmids should share at least about 500 bp of sequence overlap. Each plasmid, as desired, may be independently manipulated, followed by cotransfection in a competent host, providing complementing genes as appropriate, or the appropriate transcription factors for initiation of transcription from a target cell-specific TRE for propagation of the adenovirus. Plasmids are generally introduced into a suitable host cell such as 293 cells using appropriate means of transduction, such as cationic liposomes. Alternatively, *in vitro* ligation of the right and left-hand portions of the adenovirus genome can also be used to construct recombinant adenovirus derivative containing all the replication-essential portions of adenovirus genome. Berkner et al. (1983) *Nucleic Acid Research* 11: 6003-6020; Bridge et al. (1989) *J. Virol.* 63: 631-638.

For convenience, plasmids are available that provide the necessary portions of adenovirus. Plasmid pXC.1 (McKinnon (1982) *Gene* 19:33-42) contains the wild-type left-hand end of Ad5. pBHG10 (Bett et al. (1994); Microbix Biosystems Inc., Toronto) provides the right-hand end of Ad5, with a deletion in E3. The deletion in E3 provides 5 room in the virus to insert a 3 kb target cell-specific TRE without deleting the endogenous enhancer/promoter. The gene for E3 is located on the opposite strand from E4 (r-strand). pBHG11 provides an even larger E3 deletion (an additional 0.3 kb is deleted). Bett et al. (1994). Alternatively, the use of pBHGE3 (Microbix Biosystems, Inc.) provides the right hand end of Ad5, with a full-length of E3.

10 For manipulation of the early genes, the transcription start site of Ad5 E1A is at 498 and the ATG start site of the E1A coding segment is at 560 in the virus genome. This region can be used for insertion of a target cell-specific TRE. A restriction site may be introduced by employing polymerase chain reaction (PCR), where the primer that is employed may be limited to the Ad5 genome, or may involve a portion of the plasmid 15 carrying the Ad5 genomic DNA. For example, where pBR322 is used, the primers may use the EcoRI site in the pBR322 backbone and the XbaI site at nt 1339 of Ad5. By carrying out the PCR in two steps, where overlapping primers at the center of the region introduce a nucleotide sequence change resulting in a unique restriction site, one can provide for insertion of target cell-specific TRE at that site.

20 A similar strategy may also be used for insertion of a target cell-specific TRE element to regulate E1B. The E1B promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box. This region extends from Ad5 nt 1636 to 1701. By insertion of a target cell-specific TRE in this region, one can provide for cell-specific transcription of the E1B gene. By employing the left-hand region modified with the cell-specific response element regulating E1A, as the template for introducing a target cell-specific TRE to regulate E1B, the resulting adenovirus vector will be dependent upon the cell-specific transcription factors for expression of both E1A and E1B. In additional 25 embodiments, the 19-kDa region of E1B is deleted.

Similarly, a target cell-specific TRE can be inserted upstream of the E2 gene to 30 make its expression cell-specific. The E2 early promoter, mapping in Ad5 from 27050-27150, consists of a major and a minor transcription initiation site, the latter accounting for about 5% of the E2 transcripts, two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site (for a detailed review of

the E2 promoter architecture see Swaminathan et al., *Curr. Topics in Micro. and Immunol.* (1995) 199(part 3):177-194.

The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable for genetic manipulation. However, the E2 5 early promoter overlaps only for a few base pairs with sequences coding for a 33 kD protein on the counterstrand. Notably, the SpeI restriction site (Ad5 position 27082) is part 10 of the stop codon for the above mentioned 33 kD protein and conveniently separates the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor binding sites E2F and ATF. Therefore, insertion of a target 15 cell-specific TRE having SpeI ends into the SpeI site in the 1-strand would disrupt the endogenous E2 early promoter of Ad5 and should allow target cell-restricted expression of E2 transcripts.

For E4, one must use the right hand portion of the adenovirus genome. The E4 transcription start site is predominantly at about nt 35605, the TATA box at about nt 35631 15 and the first AUG/CUG of ORF I is at about nt 35532. Virtanen et al. (1984) *J. Virol.* 51: 822-831. Using any of the above strategies for the other genes, a UP-TRE may be 20 introduced upstream from the transcription start site. For the construction of full-length adenovirus with a target cell-specific TRE inserted in the E4 region, the co-transfection and homologous recombination are performed in W162 cells (Weinberg et al. (1983) *Proc. Natl. Acad. Sci.* 80:5383-5386) which provide E4 proteins *in trans* to complement defects 25 in synthesis of these proteins.

Adenoviral constructs containing an E3 region can be generated wherein 25 homologous recombination between an E3-containing adenoviral plasmid, for example, BHGE3 (Microbix Biosystems Inc., Toronto) and a non-E3-containing adenoviral plasmid, is carried out.

Alternatively, an adenoviral vector comprising an E3 region can be introduced into 30 cells, for example 293 cells, along with an adenoviral construct or an adenoviral plasmid construct, where they can undergo homologous recombination to yield adenovirus containing an E3 region. In this case, the E3-containing adenoviral vector and the adenoviral construct or plasmid construct contain complementary regions of adenovirus, for example, one contains the left-hand and the other contains the right-hand region, with sufficient sequence overlap as to allow homologous recombination.

Alternatively, an E3-containing adenoviral vector of the invention can be constructed using other conventional methods including standard recombinant methods (e.g., using restriction nucleases and/or PCR), chemical synthesis, or a combination of any of these. Further, deletions of portions of the E3 region can be created using standard 5 techniques of molecular biology.

Insertion of an IRES into a vector is accomplished by methods and techniques that are known in the art and described herein *supra*, including but not limited to, restriction enzyme digestion, ligation, and PCR. A DNA copy of an IRES can be obtained by chemical synthesis, or by making a cDNA copy of, for example, a picornavirus IRES. See, 10 for example, Duke et al. (1995) *J. Virol.* 66(3):1602-9) for a description of the EMCV IRES and Huez et al. (1998), *Mol. Cell. Biol.* 18(11):6178-90) for a description of the VEGF IRES. The internal translation initiation sequence is inserted into a vector genome at a site such that it lies upstream of a 5'-distal coding region in a multicistronic mRNA. For example, in a preferred embodiment of an adenovirus vector in which production of a 15 bicistronic E1A-E1B mRNA is under the control of a target cell-specific TRE, the E1B promoter is deleted or inactivated, and an IRES sequence is placed between E1A and E1B. IRES sequences of cardioviruses and certain aphthoviruses contain an AUG codon at the 3' end of the IRES that serves as both a ribosome entry site and as a translation initiation site. Accordingly, this type of IRES is introduced into a vector so as to replace the translation 20 initiation codon of the protein whose translation it regulates. However, in an IRES of the enterovirus/rhinovirus class, the AUG at the 3' end of the IRES is used for ribosome entry only, and translation is initiated at the next downstream AUG codon. Accordingly, if an enterovirus/rhinovirus IRES is used in a vector for translational regulation of a downstream 25 coding region, the AUG (or other translation initiation codon) of the downstream gene is retained in the vector construct.

Methods of packaging polynucleotides into adenovirus particles are known in the art and are also described in co-owned PCT PCT/US98/04080.

#### *Delivery of Adenovirus vectors*

The adenoviral vectors can be used in a variety of forms, including, but not limited 30 to, naked polynucleotide (usually DNA) constructs. Adenoviral vectors can, alternatively, comprise polynucleotide constructs that are complexed with agents to facilitate entry into cells, such as cationic liposomes or other cationic compounds such as polylysine; packaged into infectious adenovirus particles (which may render the adenoviral vector(s) more

immunogenic); packaged into other particulate viral forms such as HSV or AAV; complexed with agents (such as PEG) to enhance or dampen an immune response; complexed with agents that facilitate *in vivo* transfection, such as DOTMATM, DOTAPTM, and polyamines.

5        If an adenoviral vector comprising an adenovirus polynucleotide is packaged into a whole adenovirus (including the capsid), the adenovirus itself may also be selected to further enhance targeting. For example, adenovirus fibers mediate primary contact with cellular receptor(s) aiding in tropism. See, e.g., Amberg et al. (1997) *Virol.* 227:239-244. If a particular subgenus of an adenovirus serotype displayed tropism for a target cell type 10 and/or reduced affinity for non-target cell types, such subgenus(or subgenera) could be used to further increase cell-specificity of cytotoxicity and/or cytolysis.

15       The adenoviral vectors may be delivered to the target cell in a variety of ways, including, but not limited to, liposomes, general transfection methods that are well known in the art, such as calcium phosphate precipitation, electroporation, direct injection, and intravenous infusion. The means of delivery will depend in large part on the particular adenoviral vector (including its form) as well as the type and location of the target cells (i.e., whether the cells are *in vitro* or *in vivo*).

20       If used in packaged adenoviruses, adenovirus vectors may be administered in an appropriate physiologically acceptable carrier at a dose of about  $10^4$  to about  $10^{14}$ . The multiplicity of infection will generally be in the range of about 0.001 to 100. If administered as a polynucleotide construct (i.e., not packaged as a virus) about 0.01  $\mu$ g to about 1000  $\mu$ g of an adenoviral vector can be administered. The adenoviral vector(s) may be administered one or more times, depending upon the intended use and the immune response potential of the host or may be administered as multiple, simultaneous injections. 25       If an immune response is undesirable, the immune response may be diminished by employing a variety of immunosuppressants, so as to permit repetitive administration, without a strong immune response. If packaged as another viral form, such as HSV, an amount to be administered is based on standard knowledge about that particular virus (which is readily obtainable from, for example, published literature) and can be determined 30 empirically.

*Methods using the adenovirus vectors of the invention*

The subject vectors can be used for a wide variety of purposes, which will vary with the desired or intended result. Accordingly, the present invention includes methods using the adenoviral vectors described above.

5 In one embodiment, methods are provided for conferring selective cytotoxicity in cells that allow a target cell-specific TRE to function, preferably target cells, comprising contacting such cells with an adenovirus vector described herein. Cytotoxicity can be measured using standard assays in the art, such as dye exclusion, <sup>3</sup>H-thymidine incorporation, and/or lysis.

10 In another embodiment, methods are provided for propagating an adenovirus specific for cells which allow a target cell-specific TRE to function, preferably target cells, preferably cancer cells. These methods entail combining an adenovirus vector with the cells, whereby said adenovirus is propagated.

15 Another embodiment provides methods for killing cells that allow a target cell-specific TRE to function in a mixture of cells, comprising combining the mixture of cells with an adenovirus vector of the present invention. The mixture of cells is generally a mixture of normal cells and cancerous cells that allow a target cell-specific TRE to function, and can be an *in vivo* mixture or *in vitro* mixture.

20 The invention also includes methods for detecting cells which allow a target cell-specific TRE to function, such as cancer cells, in a biological sample. These methods are particularly useful for monitoring the clinical and/or physiological condition of an individual (i.e., mammal), whether in an experimental or clinical setting. In one method, cells of a biological sample are contacted with an adenovirus vector, and replication of the adenoviral vector is detected. Alternatively, the sample can be contacted with an 25 adenovirus in which a reporter gene is under control of a target cell-specific TRE. When such an adenovirus is introduced into a biological sample, expression of the reporter gene indicates the presence of cells that allow a target cell-specific TRE to function.

30 Alternatively, an adenovirus can be constructed in which a gene conditionally required for cell survival is placed under control of a target cell-specific TRE. This gene may encode, for example, antibiotic resistance. Later the biological sample is treated with an antibiotic. The presence of surviving cells expressing antibiotic resistance indicates the presence of cells capable of target cell-specific TRE function. A suitable biological sample is one in which cells that allow a target cell-specific TRE to function, such as cancer cells, may be or

are suspected to be present. Generally, in mammals, a suitable clinical sample is one in which cancerous cells that allow a target cell-specific TRE to function, such as carcinoma cells, are suspected to be present. Such cells can be obtained, for example, by needle biopsy or other surgical procedure. Cells to be contacted may be treated to promote assay conditions, such as selective enrichment, and/or solubilization. In these methods, cells that allow a target cell-specific TRE to function can be detected using *in vitro* assays that detect adenoviral proliferation, which are standard in the art. Examples of such standard assays include, but are not limited to, burst assays (which measure virus yield) and plaque assays (which measure infectious particles per cell). Propagation can also be detected by measuring specific adenoviral DNA replication, which are also standard assays.

The invention also provides methods of modifying the genotype of a target cell, comprising contacting the target cell with an adenovirus vector described herein, wherein the adenoviral vector enters the cell.

The invention further provides methods of suppressing tumor cell growth, preferably a tumor cell that allows a target cell-specific TRE to function, comprising contacting a tumor cell with an adenoviral vector of the invention such that the adenoviral vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell. For these methods, the adenoviral vector may or may not be used in conjunction with other treatment modalities for tumor suppression, such as chemotherapeutic agents (such as those listed below), radiation and/or antibodies.

The invention also provides methods of lowering the levels of a tumor cell marker in an individual, comprising administering to the individual an adenoviral vector of the present invention, wherein the adenoviral vector is selectively cytotoxic toward cells that allow a target cell-specific TRE to function. Tumor cell markers include, but are not limited to, CK-20. Methods of measuring the levels of a tumor cell marker are known to those of ordinary skill in the art and include, but are not limited to, immunological assays, such as enzyme-linked immunosorbent assay (ELISA), using antibodies specific for the tumor cell marker. In general, a biological sample is obtained from the individual to be tested, and a suitable assay, such as an ELISA, is performed on the biological sample. For these methods, the adenoviral vector may or may not be used in conjunction with other treatment modalities for tumor suppression, such as chemotherapeutic agents (such as those listed below), radiation and/or antibodies.

The invention also provides methods of treatment, in which an effective amount of an adenoviral vector(s) described herein is administered to an individual. Treatment using an adenoviral vector(s) is indicated in individuals with cancer as described above. Also indicated are individuals who are considered to be at risk for developing cancer (including 5 single cells), such as those who have had disease which has been resected and those who have had a family history of cancer. Determination of suitability of administering adenoviral vector(s) of the invention will depend, *inter alia*, on assessable clinical parameters such as serological indications and histological examination of tissue biopsies. Generally, a pharmaceutical composition comprising an adenoviral vector(s) in a 10 pharmaceutically acceptable excipient is administered. Pharmaceutical compositions are described above. For these methods, the adenoviral vector may or may not be used in conjunction with other treatment modalities for tumor suppression, such as chemotherapeutic agents (such as those listed below), radiation and/or antibodies.

15 The amount of adenoviral vector(s) to be administered will depend on several factors, such as route of administration, the condition of the individual, the degree of aggressiveness of the disease, the particular target cell-specific TRE employed, and the particular vector construct (i.e., which adenovirus gene(s) is under target cell-specific TRE control) as well as whether the adenoviral vector is used in conjunction with other treatment modalities.

20 If administered as a packaged adenovirus, from about  $10^4$  to about  $10^{14}$ , preferably from about  $10^4$  to about  $10^{12}$ , more preferably from about  $10^4$  to about  $10^{10}$ . If administered as a polynucleotide construct (i.e., not packaged as a virus), about 0.01  $\mu$ g to about 100  $\mu$ g can be administered, preferably 0.1  $\mu$ g to about 500  $\mu$ g, more preferably about 0.5  $\mu$ g to about 200  $\mu$ g. More than one adenoviral vector can be administered, either 25 simultaneously or sequentially. Administrations are typically given periodically, while monitoring any response. Administration can be given, for example, intratumorally, intravenously or intraperitoneally.

30 The adenoviral vectors of the invention can be used alone or in conjunction with other active agents, such as chemotherapeutics, that promote the desired objective. Examples of chemotherapeutics which are suitable for suppressing bladder tumor growth are BGC (bacillus Calmett-Guerin); mitomycin-C; cisplatin; thioguanine; doxorubicin; methotrexate; paclitaxel (TAXOL<sup>TM</sup>); ifosfamide; gallium nitrate; gemcitabine;

carboplatin; cyclophosphamid; vinblastine; vincristin; fluorouracil; etoposide; bleomycin. Examples of combination therapies include (CISCA (cyclophosphamide, doxorubicin, and cisplatin); CMV (cisplatin, methotrexate, vinblastine); MVMJ (methotrexate, vinblastine, mitoxantrone, carboplatin); CAP (cyclophosphamide, doxorubicin, cisplatin); MVAC (methotrexate, vinblastine, doxorubicin, cisplatin). Radiation may also be combined with a chemotherapeutic agent(s), for example, radiation with cisplatin. Administration of the chemotherapeutic agents is generally intravesical (directly into the bladder) or intravenous.

5 The following examples are provided to illustrate but not limit the invention.

10

## EXAMPLES

### **Example 1: Construction of a replication-competent adenovirus vector comprising an AFP-TRE and an EMCV IRES**

The encephalomyocarditis virus (EMCV) IRES as depicted in Table 1 was introduced between the E1A and E1B regions of a replication-competent adenovirus vector 15 specific for cells expressing AFP as follows. Table 1 shows the 519 base pair IRES segment which was PCR amplified from Novagen's pCITE vector by primers A/B as listed in Table 4. A 98 base pair deletion in the E1A promoter region was created in PXC.1, a plasmid which contains the left-most 16 mu of Ad5. Plasmid pXC.1 (McKinnon (1982) *Gene* 19:33-42) contains the wild-type left-hand end of Ad5, from Adenovirus 5 nt 22 to 20 5790 including the inverted terminal repeat, the packaging sequence, and the E1a and E1b genes in vector pBR322. pBHG10 (Bett. et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806; Microbix Biosystems Inc., Toronto) provides the right-hand end of Ad5, with a deletion in E3. The resultant plasmid, CP306 (PCT/US98/16312), was used as the backbone in overlap PCR to generate CP624. To place a Sal1 site between E1a and E1b, 25 primers C/D, E/F (Table 4) were used to amplify CP306, plasmid derived from pXC.1 and lacking the E1a promoter. After first round PCR using CP306 as template and primers C/D, E/F, the resultant two DNA fragments were mixed together for another round of overlapping PCR with primers C/F. The overlap PCR product was cloned by blunt end ligation to vector. The resultant plasmid, CP624 (Table 5), contains 100 bp deletion in 30 E1a/E1b intergenic region and introduces Sal1 site into the junction. On this plasmid, the endogenous E1a promoter is deleted, and the E1a polyadenylation signal and the E1b promoter are replaced by the Sal1 site. Next, the Sal1 fragment of CP625 was cloned into the Sal1 site in CP624 to generate CP627 (Table 5). CP627 has an EMCV IRES connecting

adenovirus essential genes E1a and E1b. In CP627, a series of different tumor-specific promoters can be placed at the *PinAl* site in front of E1a to achieve transcriptional control on E1 expression.

Table 4

Primer	Sequence	Note
A.	5'-GACGTCGACTAATTCCGGTTATTTCCA	For PCR EMCV IRES, <i>GTCGAC</i> is a SalI site.
B.	5'-GACGTCGACATCGTGTTCAAAGGAA	For PCR EMCV IRES, <i>GTCGAC</i> is a SalI site.
C.	5'-CCTGAGACGCCGACATCACCTGTG	Ad5 sequence to 1314 to 1338.
D.	5'- <u>GTCGACCATT</u> <i>CAGCAAACAAAGGCGTTAAC</i>	Antisense of Ad5 sequence 1572 to 1586. <i>GTCGAC</i> is a SalI site. Underline region overlaps with E.
E.	5'- <u>TGCTGAATGGTCGACATGGAGGCTTGGGAG</u>	Ad5 sequence 1714 to 1728. <i>GTCGAC</i> is a SalI site. Underline region overlaps with D.
F.	5'-CACAAACCGCTCTCCACAGATGCATG	Antisense of Ad5 sequence 2070 to 2094.

5

For generating a liver cancer-specific virus, an about 0.8kb AFP promoter fragment as shown in Table 3 was placed into the *PinAl* site of CP627 thereby yielding plasmid CP686. Full-length viral genomes were obtained by recombination between CP686 and a plasmid containing a right arm of an adenovirus genome. The right arms used in virus 10 recombination were pBHGE3 (Microbix Biosystems Inc.), containing an intact E3 region, and pBHG11 or pBHG10 (Bett et al. (1994) containing a deletion in the E3 region.

The virus obtained by recombination of CP686 with a right arm containing an intact E3 region was named CV890. The virus obtained by recombination of CP686 with a right arm containing a deleted E3 region (pBHG 10) was named CV840. The structure of all 15 viral genomes was confirmed by conducting PCR amplifications that were diagnostic for the corresponding specific regions.

Therefore, adenovirus vector designated CV890 comprises 0.8 kb AFP promoter, E1A, a deletion of the E1A promoter, EMCV IRES, E1B a deletion of the E1B promoter and an intact E3 region. Adenovirus vector CV840 comprises AFP promoter, E1A, a 20 deletion of the E1A promoter, EMCV IRES, E1B, a deletion of the E1B promoter and a deleted E3 region.

Table 5

Plasmid designation	Brief description
CP306	An E1A promoter deleted plasmid derived from pXC.1

CP624	Overlap PCR product from CP306 to generate 100 bp deletion and introduce a Sal1 site at E1A and E1B junction; E1A and E1B promoter deleted in E1A/E1B intergenic region.
CP625	EMCV IRES element ligated to PCR-blunt vector (Invitrogen pCR® blunt vector).
CP627	IRES element derived from CP625 by Sal1 digestion and ligated to CP624 Sal1 site placing IRES upstream from E1B.
CP628	Probasin promoter derived from CP251 by <i>Pin</i> Al digestion and cloned into <i>Pin</i> Al site on CP627.
CP629	HCMV IE promoter amplified from pCMV beta (Clontech) with <i>Pin</i> Al at 5' and 3' ends ligated into CP627 <i>Pin</i> Al site.
CP630	A 163 bp long VEGF IRES fragment (Table 1) cloned into the Sal1 site on CP628.
CP686	AFP promoter from CP219 digested with <i>Pin</i> Al and cloned into <i>Pin</i> Al site on CP627.

**Example 2: Construction of a replication-competent adenovirus vector with a probasin TRE and an EMCV IRES**

The probasin promoter as shown in Table 3 was inserted at the *Pin*Al site of 5 plasmid CP627 (see Example 1) to generate CP628, which contains a probasin promoter upstream of E1A and an EMCV IRES between E1A and E1B. Full-length viral genomes were obtained by recombination between CP628 and a plasmid containing a right arm of an adenovirus genome. The right arms used in virus recombination were pBHGE3, containing 10 an intact E3 region, and pBHG11 or pBHG10 containing a deletion in the E3 region. The structure of all viral genomes was confirmed by conducting PCR amplifications that were diagnostic for the corresponding specific regions.

Therefore, adenovirus designated CV 834 comprises probasin promoter, E1A, a deletion of the E1A promoter, EMCV IRES, E1B, a deletion of the E1B endogenous promoter and a deleted E3 region.

15

**Example 3: Construction of a replication-competent adenovirus vector with a hCMV-TRE and an EMCV IRES**

The hCMV immediate early gene (IE) promoter from plasmid CP629, originally derived from pCMVBeta (Clonetech, Palo Alto) was inserted at the *Pin*Al site of plasmid 20 CP627 (see Example 1) to generate CP629, containing a CMV IE promoter upstream of E1A and an IRES between E1A and E1B. Full-length viral genomes were obtained by recombination between CP629 and a plasmid containing a right arm of an adenovirus

genome. The right arms used in virus recombination were pBHGE3, containing an intact E3 region, and pBHG11 or pBHG10 containing a deletion in the E3 region. The structure of all viral genomes was confirmed by conducting PCR amplifications that were diagnostic for the corresponding specific regions.

5 Therefore, adenovirus vector designated CV835 comprises hCMV-IE promoter, E1A, a deletion of the E1A promoter, EMCV IRES, E1B a deletion in the E1B endogenous promoter and a deleted E3 region. CV835 lacks the hCMV enhancer and is therefore not tissue specific. By adding the hCMV IE enhancer sequence to CV835, the vector is made tissue specific.

10

**Example 4: Replication of IRES-containing adenovirus vectors with different TRES controlling E1 expression**

15 The viral replication of adenovirus vectors comprising the probasin promoter (CV836 and CV834) generally considered a weak promoter, and the human cytomegalovirus immediate early gene (HCMV-IE) promoter (CV837 and CV835), generally considered a strong promoter, were characterized in the virus yield assay.

20 Probasin promoter containing adenovirus vectors (see PCT/US98/04132), CV836 and CV834, and HCMV-IE promoter containing adenovirus vectors, CV837 and CV835, were tested against a panel of cell lines for viral replication (indicative of lethality) and specificity. Cell lines 293 (the producer line), LNCap and HepG2 were plated at  $0.5 \times 10^6$  per well in 6 well tissue culture plates, incubated for 24 hours at 37° C, then infected with CV836, CV834 or CV837 and CV835 at a multiplicity of infection (MOI) of 2 plaque forming units per cell (PFU/cell) for 4 hours at 37° C. At the end of the infection period, the medium was replaced and the cells were incubated at 37° C for a further 72 hours 25 before harvesting for a viral yield assay as described in Yu et al. (1999) *Cancer Res.* 59:1498-1504. The results are shown in Fig. 3.

30 The data demonstrate that the presence of an IRES element in the intergenic region between E1A and E1B does not significantly affect viral replication, as compared to control viruses lacking an IRES, such as a wild-type AD5 with a deletion in the E3 region. In CV834, the loss of tissue cytotoxicity could be caused by the weakness of the probasin promoter in the virus structure.

**Example 5: Comparison of dual TRE vectors with single TRE/IRES-containing vectors**

Two liver cancer-specific adenovirus vectors, CV790 and CV733 (also designated CN790 and CN733, respectively), were generated and characterized. See 5 PCT/US98/04084. These viruses contain two AFP TREs, one upstream of E1A and one upstream of E1B. They differ in that CV790 contains an intact E3 region, while the E3 region is deleted in CV733. Replication of these two viruses was compared with that of the 10 newly generated IRES-containing viruses, CV890 and CV840 (see Example 1).

Virus replication was compared, in different cell types, using a virus yield assay as 10 described in Example 4. Cells were infected with each type of virus and, 72 hrs after infection, virus yield was determined by a plaque assay. Figs. 4A and 4B show viral yield for different viruses in different cell types. The results indicate that vectors containing an 15 IRES between E1A and E1B (CV890 and CV840), in which E1B translation is regulated by the IRES, replicate to similar extents as normal adenovirus and viruses with dual AFP 20 TREs, in AFP-producing cells such as 293 cells and hepatoma cells. In SK-Hep-1 (liver cells), PA-1 (ovarian carcinoma) and LNCaP cells (prostate cells) the IRES-containing viruses do not replicate as well as dual TRE or wild-type adenoviruses, indicating that the IRES-containing viruses have higher specificity for hepatoma cells. Based on these results, it is concluded that IRES-containing vectors have unaltered replication levels, but are more 25 stable and have better target cell specificity, compared to dual-TRE vectors.

**Example 6: Uroplakin adenoviral constructs containing an EMCV IRES**

A number of E3-containing viral constructs were prepared which contained 25 uroplakin II sequences (mouse and/or human) as well as an EMCV internal ribosome entry site (IRES). The viral constructs are summarized in Table 6. All of these vectors lacked an E1A promoter and retained the E1A enhancer.

The 519 base pair EMCV IRES segment was PCR amplified from Novagen's pCITE vector by primers A/B:

primer A: 5'-GACGTCGACTAATTCCGGTTATTTCCA

30 primer B 5'-GACGTCGACATCGTGTGTTCAAAGGAA (GTCGAC is a SalI site).

The EMCV IRES element was ligated to PCR blunt vector (Invitrogen pCR® blunt vector).

#### ***CP1066***

5 The 1.9kb-(-1885 to +1) fragment of mouse UPII from CP620 was digested with AflIII (blunted) and HindIII and inserted into pGL3-Basic from CP620 which had been digested with XhoI (blunted) and HindIII to generate CP1066.

#### ***CP1086***

10 The 1.9kb mouse UPII insert was digested with PinAI and ligated with CP269 (CMV driving E1A and IRES driving E1B with the deletions of E1A/E1B endogenous promoter) which was similarly cut by PinAI.

#### ***CP1087***

15 The 1kb (-1128 to +1) human UPII was digested with PinAI from CP665 and inserted into CP629 which had been cut by PinAI and purified (to elute CMV).

#### ***CP1088***

20 The 2.2kb (-2225 to +1) human UPII was amplified from CP657 with primer 127.2.1 (5'-AGGACCGGTCACTATAGGGCACGCGTGGT-3') PLUS 127.2.2 (5'-AGGACCGGTGGGATGCTGGGCTGGGAGGTGG-3') and digested with PinAI and ligated with CP629 cut with PinAI.

CP627 is an Ad5 plasmid with an internal ribosome entry site (IRES) from encephelomyocarditis virus (EMCV) at the junction of E1A and E1B. First, CP306 (Yu et al., 1999) was amplified with primer pairs 96.74.3/96.74.6 and 96.74.4/96.74.5.

25 The two PCR products were mixed and amplified with primer pairs 96.74.3 and 96.74.5. The resultant PCR product contains a 100bp deletion in E1A-E1B intergenic region and a new SalI site at the junction. EMCV IRES fragment was amplified from pCITE-3a(+) (Novagen) using primers 96.74.1 and 96.74.2. The SalI fragment containing 30 IRES was placed into SalI site to generate CP627 with the bicistronic E1A-IRES-E1B

cassette. CP629 is a plasmid with CMV promoter amplified from pCMVbeta (Clontech) with primer 99.120.1 and 99.120.2 and cloned into PinAI site of CP627.

CP657 is a plasmid with 2.2kb 5' flanking region of human UP II gene in pGL3-Basic (Promega). The 2.2kb hUPII was amplified by PCR from Genome Walker product 5 with primer 100.113.1 and 100.113.2 and TA-cloned into pGEM-T to generate CP655.

The 2.2kb insert digested from SacII (blunt-ended) and KpnI was cloned into pGL3-Basic at HindIII (blunted) and KpnI to create CP657.

#### **CP1089**

10 The 1kb (-965 to +1) mouse UPII was digested by PinAI from CP263 and inserted into CN422 (PSE driving E1A and GKE driving E1B with the deletions of E1A/E1B endogenous promoter) cut by PinAI and purified and further digested with EagI and ligated with 1kb (-1128 to +1) human UPII cut from CP669 with EagI.

#### **CP1129**

15 The 1.8kb hUPII fragment with PinAI site was amplified from CP657 with primer 127.50.1 and 127.2.2 and cloned into PinAI site of CP629.

#### **CP1131**

20 CP686 was constructed by replacing the CMV promoter in CP629 with an AFP fragment from CP219. A 1.4kb DNA fragment was released from CP686 by digesting it with BssHII, filling with Klenow, then digesting with BglII. This DNA fragment was then cloned into a similarly cut CP686 to generate CP1199. In CP1199, most of the E1B 19-KDa region was deleted. The 1.8kb hUPII fragment with PinAI site was amplified from CP657 by PCR with primer 127.50.1 and 127.2.2 and inserted into similarly digested CP1199 to create CP1131.

25 The plasmids above were all co-transfected with pBHGE3 to generate CV874 (from CP1086), CV875 (from CP1087), CV876 (from 1088) and CV877 (from CP1089), CV882 (from CP1129) and CV884 (from CP1131). CP1088, CP1129 and CP1131 were cotransfected with pBHGE3 for construction of CV876, CV892 and CV884, respectively by lipofectAMINE (Gibco/BRL) for 11-14 days. pBHGE3 was purchased from 30 Microbix, Inc., and was described previously. The cells were lysed by three freeze-thaw

cycles and plaqued on 293 cells for a week. The single plaques were picked and amplified by infection in 293 cells for 3-5 days. The viral DNAs were isolated from the lysates and the constructs were confirmed by PCR with primer 31.166.1/ 51.176 for CV876 and primer 127.50.1/51.176 for CV882 and CV884 at E1 region and primer 32.32.1/2 for all three viruses at E3 region.

TABLE 6

Name	Vector	Ad 5 Vector	E1A TRE	E1B TRE	E3
CV874	CP1086	pBHGE3	1.9 kb mUPII	IRES	intact
CV875	CP1087	pBHGE3	1.0 kb hUPII	IRES	intact
CV876	CP1088	pBHGE3	2.2 kb hUPII	IRES	intact
CV877	CP1089	pBHGE3	1.0 kb mUPII	1.0 kb hUPII (E1B promoter deleted)	intact
CV882	CP1129	pBHGE3	1.8 kb hUPII	IRES	intact
CV884	CP1131	pBHGE3	1.8 kb hUPII	IRES (E1B 19-kDa deleted)	intact

Viruses are tested and characterized as described above.

Primer sequences:

96.74.1	GACGTCGACATCGTGTTCAAAGGAA
96.74.2	GACGTCGACTAATTCCGGTTATTTCCA
96.74.3	CCTGAGACGCCGACATCACCTGTG
96.74.4	TGCTGAATGGTCGACATGGAGGCTTGGGAG
96.74.5	CACAACCGCTCTCCACAGATGCATG
96.74.6	GTCGACCATTCAAGCAAACAAAGGCGTTAAC
100.113.1	AGGGGTACCCACTATAGGGCACGCGTGGT
100.113.2	ACCCAAGCTTGGGATGCTGGGCTGGGAGGTGG
127.2.2	AGGACCGGTGGGATGCTGGGCTGGGAGGTGG
127.50.1	AGGACCGGTCAAGCTTACCCCCAGACCCAC
31.166.1	TGCGCCGGTGTACACAGGAAGTGA
32.32.1	GAGTTTGTGCCATCGGTCTAC
32.32.2	AATCAATCCTTAGTCCTCCTG
51.176	GCAGAAAAATCTTCCAAACACTCCC
99.120.1	ACGTACACCGGTGTTACATAACTTAC
99.120.2	CTAGCAACCGGTGGTTACTAAACG

**Example 7: Construction of a replication-competent adenovirus vector with a tyrosinase TRE and EMCV IRES**

CP621 is a plasmid containing a human tyrosinase enhancer and promoter elements in a PinAI fragment. This fragment is ligated to the PinAI site on CP627 to generate

5 CP1078. CP1078 is combined with pBHGE3 to generate a new melanoma specific virus, CV859. Table 3 depicts the polynucleotide sequence of the PinAI fragment which contains a tyrosinase promoter and enhancer.

**Example 8: Construction of a replication-competent adenovirus vector with a 10 probasin-TRE and a VEGF IRES**

Using a strategy similar to that described in Example 1, the IRES fragment from the mouse vascular endothelial growth factor (VEGF) gene is amplified and cloned into CP628 at the Sall site. Table 1 depicts the IRES fragment obtainable from vascular endothelial growth factor (VEGF) mRNA. In order to clone this fragment into the E1a/E1b intergenic 15 region, two pieces of long oligonucleotide are synthesized. The sense oligonucleotide is shown in the Table, whereas the second piece is the corresponding antisense one. After annealing the two together to create a duplex, the duplex is subjected to Sall digestion and the resulting fragment is cloned into the Sall site on CP628. The resulting plasmid, CP630, has a probasin promoter in front of E1a and an VEGF IRES element in front of E1b. This 20 plasmid is used to construct a prostate cancer-specific virus comprising the VEGF IRES element.

**Example 9: Construction of a replication-competent adenovirus vector with an 25 AFP-TRE and a VEGF IRES**

Using a strategy similar to Example 1, a PinAI fragment which contains AFP TRE can be obtained. This AFP TRE is cloned into the PinA1 site in front of E1A on CP628 yielding plasmid CP1077. This plasmid has the AFP TRE for E1 transcriptional control and the VEGF IRES element before E1b. CP1077 can be recombined with pBHGE3 to generate a liver-specific adenovirus, designated as CV858.

**Example 10: Construction of a replication-competent adenovirus vector with a hKLK2-TRE and a EMCV IRES**

Using a strategy similar to Example 1, the TRE fragment from human glandular kallikrein II as shown in Table 3 was cloned into the PinAI site in CP627. The resultant 5 plasmid, CP1079, is cotransfected with pBHGE3 to create CV860.

**Example 11: Treatment of Hep3B Tumor Xenografts with Replication-Competent Hepatoma Specific CV790 and Doxorubicin and Hepatoma Specific CV890 and Doxorubicin**

10 CV790 is an AFP producing hepatocellular carcinoma specific adenovirus, with E1A and E1B under the control of an identical AFP promoter and enhancer (822 base pair promoter shown in Table 3) with an E3 region. The CV890 adenovirus construct is also a hepatoma or liver-specific adenoviral mutant with the E1A and E1B genes under transcriptional control of 822bp AFP promoter (827 bp including nucleotides for restriction 15 site), wherein E1B is under translational control of EMCV IRES and having an intact E3 region. The structure of CV890 therefore reads as AFP/E1A, IRES/E1B, E2, E3, E4. *In vivo* studies of the efficacy of combinations of CV790 and doxorubicin and CV890 and doxorubicin were performed according to the protocols described in detail in Example 4, with minor alterations which are described below.

20 Xenografts in the study of CV790 and CV890 combined with chemotherapeutic agents utilized liver carcinoma Hep3B cells. Virus, CV790 or CV890, was administered by a single intravenous injection of  $1 \times 10^{11}$  particles through the tail veins of the nude mice. One day after virus delivery, a single dose of doxorubicin was given to each animal by i.p. injection. The doxorubicin dose was 10 mg/kg for both doxorubicin alone and doxorubicin 25 combined with virus treatments. Tumor volume was measured once a week for six weeks. Tumors were measured weekly in two dimensions by external caliper and volume was estimated by the formula [length (mm) x width (mm)<sup>2</sup>]/2.

Figure 8 depicts the anti-tumor activity of CV890 containing an IRES as compared to CV 790 containing dual TREs. As Figure 8 demonstrates, relative tumor volume was 30 less with administration of CV890 than administration of CV790. Furthermore, both CV790/doxorubicin and CV890/doxorubicin treatment of the hepatoma showed synergistic results. Four days after treatment with either CV790/doxorubicin or CV890/doxorubicin the relative tumor volume was less than 10%. Unlike mice treated with either virus alone

or doxorubicin alone, after day 4, the relative tumor volume did not increase for either the either CV790/doxorubicin or CV890/doxorubicin treated mice. At day 6 in the control mice, the relative tumor volume was approximately 1000% in the CV790 study and approximately 600% in the CV890 study. The relative tumor volumes of mice treated with 5 virus alone were 250% (CV790) and 520% (CV890) while the relative tumor volumes for mice treated with doxorubicin alone were 450% with 280% in the CV790 study and 500% in the CV890 study.

10 **Example 12: *In vitro* characterization of melanocyte-specific TRE-containing adenoviral constructs**

An especially useful objective in the development of melanocyte cell-specific adenoviral vectors is to treat patients with melanoma. Methods are described below for measuring the activity of a melanocyte-specific TRE and thus for determining whether a given cell allows a melanocyte-specific TRE to function.

15 **Cells and Culture Methods**

Host cells such as, HepG2 (liver); Lovo (colon); LNCaP (prostate); PMEL (melanoma); SKMel (melanoma); G361 (melanoma) and MeWo cells are obtained at 20 passage 9 from the American Type Culture Collection (Rockville, MD). MeWo cells are maintained in RPMI 1640 medium (RPMI) supplemented with 10% fetal bovine serum (FBS; Intergen Corp.), 100 units/mL of penicillin, and 100 units/mL streptomycin. MeWo cells being assayed for luciferase expression are maintained in 10% strip-serum (charcoal/dextran treated fetal bovine serum to remove T3, T4, and steroids; Gemini Bioproduct, Inc., Calabasas, CA) RPMI.

25 **Transfections of MeWo Cells**

For transfections, MeWo cells are plated out at a cell density of  $5 \times 10^5$  cells per 6-cm culture dish (Falcon, NJ) in complete RPMI. DNAs are introduced into MeWo cells after being complexed with a 1:1 molar lipid mixture of N-[1-(2,3-dioleyloxy)propyl-*N,N,N*-trimethylammonium chloride (DOTAP™; Avanti Polar Lipids, AL) and dioleoyl-phosphatidylethanolamine (DOPE™; Avanti Polar Lipids, AL); DNA/lipid complexes are 30 prepared in serum-free RPMI at a 2:1 molar ratio. Typically, 8  $\mu$ g (24.2 nmole) of DNA is diluted into 200  $\mu$ L of incomplete RPMI and added dropwise to 50 nmole of transfecting, lipids in 200  $\mu$ L of RPMI with gentle vortexing to insure homogenous mixing of

components. The DNA/lipid complexes are allowed to anneal at room temperature for 15 minutes prior to their addition to MeWo cells. Medium is removed from MeWo cells and replaced with 1 mL of serum-free RPMI followed by the dropwise addition of DNA/lipid complexes. Cells are incubated with complexes for 4-5 hours at 37°C, 5% CO<sub>2</sub>. Medium 5 was removed and cells washed once with PBS. The cells were then trypsinized and resuspended in 10% strip-serum RPMI (phenol red free). Cells were replated into an opaque 96-well tissue culture plate (Falcon, NJ) at a cell density of 40,000 cells/well per 100 µL media and assayed.

Plaque assays

10 To determine whether the adenoviral constructs described above replicate preferentially in melanocytes, plaque assays are performed. Plaquing efficiency is evaluated in the following cell types: melanoma cells (MeWo), prostate tumor cell lines (LNCaP), breast normal cell line (HBL-100), ovarian tumor cell line (OVCAR-3, SK-OV-3), and human embryonic kidney cells (293). 293 cells serve as a positive control for 15 plaquing efficiency, since this cell line expresses Ad5 E1A and E1B proteins. For analyzing constructs comprising a melanocyte-specific TRE, cells that allow a melanocyte-specific TRE to function, such as the cell lines provided above and cells that do not allow such function, such as HuH7, HeLa, PA-1, or G361, are used. The plaque assay is performed as follows: Confluent cell monolayers are seeded in 6-well dishes eighteen hours before 20 infection. The monolayers are infected with 10-fold serial dilutions of each virus. After infecting monolayers for four hours in serum-free media (MEM), the medium is removed and replaced with a solution of 0.75% low melting point agarose and tissue culture media. Plaques are scored two weeks after infection.

**Example 13: Construction of a replication-competent adenovirus vector with a CEA-TRE and a EMCV IRES**

Using a strategy similar to Example 1, the TRE fragment from Carcinembryonic antigen (CEA)(Table 3, SEQ ID NO:14) is used to construct virus designated CV873. A 5 PinAI fragment containing the CEA-TRE was cloned into the PinAI site in front of E1A 10 CP627 for the transcriptional control. The resultant plasmid CP1080 is used together with pBHGE3 to generate CV873.

**Example 14: *In vitro* and *In vivo* assays of anti-tumor activity**

10 An especially useful objective in the development of urothelial cell-specific adenoviral vectors is to treat patients with bladder cancer. An initial indicator of the feasibility is to test the vector(s) for cytotoxic activity against cell lines and tumor xenografts grown subcutaneously in Balb/c nu/nu mice.

***In vitro* characterization of CV876**

15 **Virus yield assay for CV876**

5 X 10<sup>5</sup> 293, RT-4, SW780, PA-1, G361, MKNI, HBL-100, Fibroblast (from lung) and Smooth muscle cells (from bladder) were plated into each well of six-well plates. 20 Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV876 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. After an additional 72h at 37°C, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

25 Unlike wt. Ad5, CV802 which grows well in all of the cells tested, CV876 replicates much better in permissive cells (293, RT-4 and SW780) than in non-permissive cells (PA-1, G361, MKNI, HBL-100 and primary cells) by about 100-10000 fold. Noticeably, the replication in SW780 for CV876 is about 100 fold less than CV802, which indicates the limitation of this virus in efficacy.

30 **Growth curve experiment for CV876**

5 X 10<sup>5</sup> RT-4, PA-1, Smooth muscle and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml

of serum-free RPMI 1640 containing CV802 (wt.Ad5 with 133) or CV876 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. At different time points of 0, 12, 24, 36, 48, 72, 96 and 120h, the cells were scraped into medium and lysed by three freeze-thaw 5 cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Very similar as in virus yield assay, CV876 replicates well only in RT-4 but not in primary cells and PA-1 over a 120h period of time. However, CV876 does show a delay of replication in RT-4 compared to CV802.

10 Cytopathic effect assay for CV876

5 X 10<sup>5</sup>293, RT-4, SW780, PA-1, MKN1 and LNCap were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV876 at increasing MOI from 0.001 to 10 (the data shown was at MOI 1). After a 4-h incubation at 37°C, medium 15 was replaced with 3ml of complete RPMI 1640 and incubated at 37°C for 6-8 days when cytopathic effect was observed for CV802 at MOI 0.01.

CV802 shows efficacy in all the cells tested while CV876 only kills the permissive cells (293, RT-4 and SW780) but not the non-permissive cells (PA-1, MKN-1 and LNCap).

MTT assay for CV876

20 2 X 10<sup>4</sup>293, RT-4, SW780, MKN1, PA-1, HBL-100, Smooth muscle cells (from bladder) and Fibroblast (from lung) were plated into each well of 96-well plates. Twenty-four hours later, the cells were infected with CV802 and CV876 at increasing MOI from 0.001 to 10 in complete RPMI 1640. A rapid colorimetric assay for cell growth and survival was run at different time point of day 1, 3, 5, 7 and 10. The medium was replaced 25 by 50ul of MTT at 1mg/ml solution, which is converted to an insoluble purple formazan by dehydrogenase enzymes present in active mitochondria of live cells. After 3-4h incubation at 37°C, the solution was replaced by isopropanol and the plates were incubated at 30°C for 1h and read at 560nm test wavelength and 690nm reference wavelength.

Similar as the results in CPE assay, CV876 shows efficacy only in permissive cells 30 but not in non-permissive cells. Again, in RT-4 and SW780, CV876 kills the cells much slower than CV802.

*In vitro* characterization of CV882Virus yield assay for CV882

5 X 10<sup>5</sup> 293, RT-4, SW780, G361, LNCap, HBL-100, MKN1, PA-1, Fibroblast and Smooth muscle cells were plated into each well of six-well plates. Twenty-four hours later, 5 medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV882 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. After an additional 72h at 37°C, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque 10 assay for 8-10 days under semisolid agarose on 293 cells.

The replication of CV882 in permissive cells (293, RT-4 and SW780) is comparable to CV802 (the difference is less than 100 fold) while it shows over 1000-1000000 fold difference in non-permissive cells (G361, LNCap, HBL-100, MKN1, PA-1 and primary cells).

15 Growth curve experiment for CV882

5 X 10<sup>5</sup> RT-4, PA-1, and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV882 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of 20 complete RPMI 1640 were added to each well. At different time points of 0, 12, 24, 36, 48, 72, 96 and 120h, the cells were scraped into medium and lysed by three, freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Very similar as in virus yield assay, CV882 replicates well only in RT-4 but not in 25 primary cells and PA-1 over a 120h period of time. Additionally, CV882 shows better replication in RT-4 compared to CV876.

Cytopathic effect assay for CV882

5 X 10<sup>5</sup> 293, RT-4, SW780, HBL-100, G361, PA-1 and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and 30 replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV882

at increasing MOI from 0.001 to 10 (the data shown was at MOI 1). After a 4-h incubation at 37°C, medium was replaced with 3ml of complete RPMI 1640 and incubated at 37°C for 6-8 days when cytopathic effect was observed for CV802 at MOI 0.01.

CV802 shows efficacy in all the cells tested while CV882 only kills the permissive 5 cells (293, RT-4 and SW780) but not the non-permissive cells (HBL-100, G361, PA-1 and Fibroblast cells).

#### MTT assay for CV882

2 X 10<sup>4</sup> RT-4, SW780, PA-1, HBL-100, U118 and Fibroblast were plated into each well of 96-well plates. Twenty-four hours later, the cells were infected with CV802 and 10 CV882 at increasing MOI from 0.001 to 10 in complete RPMI 1640. A rapid colorimetric assay for cell growth and survival was run at different time points of day 1, 3, 5, 7 and 10. The medium was replaced by 50ul of MTT at 1mg/ml solution, which is converted to an insoluble purple formazan by dehydrogenase enzymes present in active mitochondria of live cells. After 3-4h incubation at 37°C, the solution was replaced by isopropanol and the 15 plates were incubated at 30°C for 1h and read at 560nm test wavelength and 690nm reference wavelength.

Similar as the results in CPE assay, CV882 shows efficacy only in permissive cells but not in non-permissive cells.

#### In Vitro Characterization of CV884

##### Virus yield assay for CV884

5 X 10<sup>5</sup> 293, RT-4, SW780, G361, LNCap, HBL-100, MKN1, PA-1, Fibroblast and Smooth muscle cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV984 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells 25 were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. After an additional 72h at 37°C, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

The replication of CV884 is very similar as CV802 in permissive cells (293, RT-4 30 and SW780) but shows over 1000 fold difference with CV802 in non-permissive cells

(G361, LNCap, HBL-100, MKN1, PA-1 and primary cells). CV884 shows better efficacy than CV876 and CV882 without losing much specificity.

Growth curve experiment for CV884

5 X 10<sup>5</sup>RT-4, PA-1, Smooth muscle and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV884 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. At different time points of 0, 12, 24, 36, 48, 72, 96 and 120h, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Very similar as in virus yield assay, CV884 replicates very well only in RT-4 (similar as CV802) but not in primary cells and PA-1. Again, the replication of CV884 is better than CV882 and CV876.

15 Cytopathic effect assay for CV884

5 X 10<sup>5</sup>293, RT-4, SW780, G361, PA-1 and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV884 at increasing MOI from 0.001 to 10 (the data shown was at MOI 1). After a 4-h incubation at 37°C, medium was replaced with 3ml of complete RPMI 1640 and incubated at 37°C for 6-8 days when cytopathic effect was observed for CV802 at MOI 0.01.

CV802 shows efficacy in all the cells tested while CV884 only kills the permissive cells (293, RT-4 and SW780) but not the non-permissive cells (G361, PA-1 and Fibroblast cells).

25 MTT assay for CV884

2 X 10<sup>4</sup>293, RT-4, SW780, U118, Fibroblast and Smooth muscle cells were plated into each well of 96-well plates. Twenty-four hours later, the cells were infected with CV802 and CV884 at increasing MOI from 0.001 to 10 in complete RPMI 1640. A rapid colorimetric assay for cell growth and survival was run at different time points of day 1, 3, 5, 7 and 10. The medium was replaced by 50ul of MTT at 1mg/ml solution which is

converted to an insoluble purple formazan by dehydrogenase enzymes present in active mitochondria of live cells. After 3-4h incubation at 37°C, the solution was replaced by isopropanol and the plates were incubated at 30°C for 1h and read at 560nm test wavelength and 690nm reference wavelength.

5       Similar as the results in CPE assay, CV884 shows strong efficacy (similar as wt. Ad5) only in permissive cells but not in non-permissive cells.

#### In vivo activity of CV808

Mice were given subcutaneous (SC) injections of  $1 \times 10^6$  sW780 cells. When tumors grew to about 500 mm<sup>3</sup>, CV808 was introduced into the mice ( $5 \times 10^7$  PFU of virus 10 in 0.1 ml PBS and 10% glycerol) intratumorally. Control mice received vehicle alone. Tumor sizes were measured weekly. The results are shown in FIG. 11. The data indicate that CV808 was effective at suppressing tumor growth.

While it is highly possible that a therapeutic based on the viruses described here would be given intralesionally (i.e., direct injection), it would also be desirable to 15 determine if intravenous (IV) administration of adenovirus vector can affect tumor growth. If so, then it is conceivable that the virus could be used to treat metastatic tumor deposits inaccessible to direct injection. For this experiment, groups of mice bearing bladder epithelial tumors are inoculated with  $10^8$  to  $10^{10}$  PFU of an adenoviral vector by tail vein injection, or with buffer used to carry the virus as a negative control. The effect of IV 20 injection of the adenoviral vector on tumor size is compared to vehicle treatment.

#### **Example 15: Synergistic Effect of CV 890 with Chemotherapeutics**

##### Materials and Methods

Cells. Hepatocellular carcinoma cell lines HepG2, Hep3B, PLC/PRF/5, SNU449, 25 and Sk-Hep-1, Chang liver cell (human normal liver cells), as well as other tumor cell lines PA-1 (ovarian carcinoma), UM-UC-3 (bladder carcinoma), SW 780 (bladder carcinoma), HBL100 (breast epithelia), Colo 201 (Colon adenocarcinoma), U 118 MG (glioblastoma) and LNCaP (prostate carcinoma) were obtained from the American Type Culture Collection. HuH-7 (liver carcinoma) was a generous gift of Dr. Patricia Marion (Stanford 30 University). 293 cells (human embryonic kidney containing the E1 region of Adenovirus) were purchased from Microbix, Inc. (Toronto, Canada). The primary cells nBdSMC (normal human bladder smooth muscle cells), nHLFC (normal human lung fibroblast

cells), and nHMEC (normal human mammary epithelial cells) were purchased from Clonetics (San Diego, California). All tumor cell lines were maintained in RPMI 1640 (BioWhittaker, Inc.) supplemented with 10% fetal bovine serum (Irvine Scientific), 100 U/ml penicillin and 100 ug/ml streptomycin. Primary cells were maintained in accordance 5 with vendor instructions (Clonetics, San Diego). Cells were tested for the expression of AFP by immunoassay (Genzyme Diagnostics, San Carlos, CA).

Virus yield and one-step growth curves. Six well dishes (Falcon) were seeded with 5x10<sup>5</sup> cells per well of calls of interest 24 hrs prior to infection. Cells were infected at an 10 multiplicity of infection (MOI) of 2 PFU/cell for three hours in serum-free media. After 3 hours, the virus containing media was removed, monolayers were washed three times with PBS, and 4 ml of complete media (RPMI1640 + 10% FBS) was added to each well. 72 hours post infection, cells were scraped into the culture medium and lysed by three cycles of freeze-thaw.

The one-step growth curves time points were harvested at various time points after 15 infection. Two independent infections of each virus cell-combination were titered in duplicate on 293 cells (Yu et al., 1999, *Cancer Research*, 59:1498-1504).

Northern blot analysis. Hep3B or HBL100 cells were infected at an MOI of 20 PFU/cell (plaque forming unit per cell) with either CV802 or CV890 and harvested 24 hours post infection. Total cell RNA was purified using the RNeasy protocol (Qiagen). 20 The Northern blot was conducted using NorthernMax Plus reagents (Ambion, Austin, Texas). 5ug of RNA was fractionated on a 1% agarose, formaldehyde-based denaturing gel and transferred to a BrightStar-Plus (Ambion) positively charged membrane by capillary transfer. The antisense RNA probes for E1A (adenovirus genome 501bp to 1141bp) or E1B (1540bp-3910bp) were PCR products cloned in pGEM-T easy (Promega) and 25 transcription labeled with [ $\alpha$  <sup>32</sup>P] UTP. Blots were hybridized at 68°C for 14 hours with ZipHyb solution and washed using standard methods (Ambion). Membranes were exposed to BioMax film (Kodak).

Western blot analysis. Hep3B or HBL100 cells were infected at MOI of 20 PFU/cell with either CV802 or CV890 and harvested 24 hours post infection. Cells were 30 washed with cold PBS and lysed for 30 min on ice in (50mM Tris, pH8.0, 150 mM NaCl, 1% IGEPAL CA360 a NP40 equivalent (Sigma), 0.5% sodium deoxycholate, and protease inhibitor cocktail from (Roche, Palo Alto, California). After 30 min centrifugation at 4C, the supernatant was harvested and the protein concentration determined with protein assay

ESL kit (Roche). Fifty micrograms of protein per lane were separated on 816% SDS-PAGE and electroblotted onto Hybond ECL membrane (Amersham Pharmacia, Piscataway, New Jersey). The membrane was blocked overnight in PBST (PBS with 0.1% Tween-20) supplemented with 5% nonfat dry milk. Primary antibody incubation was done 5 at room temperature for 2-3 hrs with PBST/1 % milk diluted antibody, followed by wash and 1 hr incubation with diluted horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California). Enhanced chemiluminescence (ECL; Amersham Pharmacia) was used for the detection. E1A antibody (clone M58) was from NeoMarkers (Fremont, California), E1B-21 kD antibody was from Oncogene 10 (Cambridge, Massachusetts). All antibodies were used according manufacturer's instruction.

Cell viability assay and statistical analysis. To determine the cell killing effect of virus and chemotherapeutic agent in combination treatment, a cell viability assay was conducted as previously described with modifications (Denizot, 1986, Journal 15 Immunology. Methods, 89:271-277). On 96 well plates, cells of interest were seeded at 10,000 calls per well 48 hr prior to infection. Cells were then treated with virus alone, drug alone, or in combination. Cell viability was measured at different time points by removing the media, adding 50  $\mu$ l of 1mg/ml solution of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl- $^2$ H-tetrazolium bromide) (Sigma, St. Louis, MO) and incubating for 3 hrs at 37°C. 20 After removing the MTT solution, the crystals remaining in the wells were solubilized by the addition of 50  $\mu$ l of isopropanol followed by 30C incubation for 0.5 hr. The absorbency was determined on a microplate reader (Molecular Dynamics) at 560 nm (test wavelength) and 690 nm (reference wavelength). The percentage of surviving cells was estimated by dividing the OD<sub>550</sub>-OD<sub>650</sub> of virus or drug treated cells by the OD<sub>550</sub>-OD<sub>650</sub> of control cells.

one week prior to tumor implantation. Xenografts were established by injecting  $1 \times 10^6$  Hep3B, HepG2 or LNCaP cells suspended in 100 $\mu$ l of RPMI 1640 media subcutaneously. When tumors reached between 200 mm<sup>3</sup> and 300 mm<sup>3</sup>, mice were randomized and dosed with 100  $\mu$ l of test article via intratumoral or the tail vein injection. Tumors were measured 5 in two dimensions by external caliper and volume was estimated by the formula [length (mm) x width (mm)<sup>2</sup>]/2. Animals were humanely killed when their tumor burden became excessive. Serum was harvested weekly by retro-orbital bleed. The level of AFP in the serum was determined by AFP Immunoassay kit (Genzyme Diagnostics, San Carlos, CA). The difference in mean tumor volume and mean serum AFP concentration between 10 treatment groups was compared for statistical significance using the unpaired, two-tailed, *t*-test.

Transcription and Translation of E1A/E1B Bicistronic Cassette of CV890 in Different Cells.

In wild type adenovirus infection, E1A and E1B genes produce a family of 15 alternatively spliced products. It has been found that there are five E1A mRNAs, among them 12S (880 nucleotides, nts) and 13S (1018 nts) mRNAs are the dominant ones that are expressed both early and late after infection. The 12S and 13S mRNAs encode the gene product of 243 amino acids (243R) and 289 amino acids (289R) respectively (reviewed by Shenk, 1996). The two major E1B transcripts that code for 19kD and 55kD proteins are 20 12S (1031 nts) and 22S (2287 nts) mRNAs. E1B 12S mRNA only codes the 19kD product, whereas the 22S mRNA codes for both 19kD and 55kD products due to different initiation sites during translation. In the current study, the generation of E1A-IRES-E1B bicistronic cassette was expected to change the pattern of E1A and E1B transcripts in viral infection. Therefore, Northern blot analysis was conducted to evaluate the steady-state level of E1A 25 and E1B transcripts. First, CV802 or CV890 were infected to Hep3B (AFP) or HBL100 (AFP) cells for 24 hours. The total RNA samples were separated on agarose gels and processed for Northern blot by hybridizing to antisense RNA probes. The Northern blot with E1A probe visualized the 12S and 13S mRNAs in both wild type CV802 infected cells. For CV890, E1A transcripts can only be seen in Hep3B cells, indicating the 30 conditional transcription of E1A. It is of interest to find that in CV890, there is only one large transcript (about 3.51Kb), whereas the 12S and 13S mRNAs are no longer present. This large transcript indicates the continuous transcription of E1A-IRES-E1B bicistronic cassette, suggesting an alteration of viral E1A splicing pattern in CV890. Transcription of

E1B from CV890 also appears to be AFP-dependent. It is clear that both 12S and 22S mRNAs of E1B were present in wild type CV802 samples, whereas the 128 mRNA and an enlarged 22S mRNA (3.5Kb) appeared in CV890 infected cells. Obviously, the identity of this enlarged mRNA is the same 3.5Kb transcript as visualized in E1A blot, which is from 5 the transcription of E1A/E1B bicistronic cassette. Therefore, the E1B mRNA is tagged after E1A mRNA in this large transcript. This large transcript contains all the coding information for E1A, E1B 19kD and E1B 55kD. The mRNA splice pattern that appears in CV802 is not valid in CV890, the 12S mRNA with E1B probe disappeared. Meanwhile, in the E1B Northern blot, due to the selection of our E1B probe (1540bp-3910bp), mRNA of 10 the Adenovirus gene IX (3580bp-4070bp), the hexon-associated protein, was also detected. In CV890 infected Hep3B cells, gene IX expression is equivalent to that of CV802, whereas in CV890 infected HBL100, its expression was also completely shut down. This result further demonstrated that the AFP controlled E1A/E1B expression is the key for late gene expression as well as viral replication.

15 Results of the same samples in the Western blot also indicate that CV890 has AFP dependent expression of E1A and E1B. Under our experimental conditions, E1A expression level of CV890 in Hep3B cells is similar to that of CV802. However, when E1B 19kD protein was detected, it was found that the expression level was much lower than CV802 E1A. Previously, it has been addressed that IRES-mediated second gene has 20 less expression (Mizuguchi et al., 2000, *Mol. Ther.* 1:376-382). Taken together, CV890 infection in permissive Hep3B cells can produce normal amounts of E1A and lesser amounts of E1B proteins capable of initiating a normal productive infection. In AFP<sup>+</sup> cells, however, this process was attenuated due to a lack of E1A and E1B gene transcription and translation. These data demonstrated that the expression of both E1A and E1B genes are 25 under the control of AFP TRE and the artificial E1A/E1B bicistronic cassette is functioning properly in CV890.

In Vitro Replication Specificity of CV890 in Tumor Cells and Primary Cells.

From *in vitro* comparison of virus yield, CV890 has a better specificity profile than CV732 (CV732 is an AFP-producing, cell-specific adenovirus variant in which the E1A 30 gene is under control of AFP-TRE). In order to gain further insights of using CV890 in liver cancer therapy, more tumor cell lines and primary cells were tested to characterize *in vitro* virus replication. First, all cells in the study were analyzed for their AFP status by AFP immune assay. Based on AFP produced in the cells and media, all the cells were

divided into three groups, high ( $>2.5 \mu\text{g}/10^6 \text{ cells}/10 \text{ days}$ ), low ( $<0.6 \mu\text{g}/10^6 \text{ cells}/10 \text{ days}$ ) and none (undetectable in our study) (Table 7). It was confirmed that replication of CV890 in different cell lines correlates well with the AFP status of the host cell. Among the group of liver cell lines, CV890 only replicates well in AFP<sup>+</sup> cells, including Hep3B, HepG2, 5 Huh7, SNU449 and PLC/PRF/5. The amount of AFP required for the promoter activity seems very low as one of the hepatoma cell lines, SNU449, a previous reported AFP<sup>-</sup> cell (Park et al., 1995, *Int. J. Cancer* 62:276-282), produces very low AFP (about 60 ng/ $10^6$  cells/10 days) compared to other cells. Nevertheless, even with very low amount of AFP, SNU449 cells can still support CV890 replication to the extent comparable to cells 10 producing significantly higher levels of AFP such as HepG2. Compared to CV802, CV890 is attenuated 5,000 to 100,000 fold in cells that do not produce AFP, including the hepatoma cell Sk-Hepl and Chang liver cell, other tumor cells and primary cells. Taken together the results indicate that CV890 has shown a good specificity profile from a broad 15 spectrum of tumor cells. Among them, only the AFP<sup>+</sup> liver cells, AFP production level from high to low, are permissive for CV890.

In another experiment, CV890 was compared to CV802 for their single step growth curves on different cells. Results demonstrated that CV890 has a similar growth kinetics to wild-type CV802 in AFP<sup>+</sup> cells except that virus yields are slightly lower (2-8 fold) in low AFP producing cells. In consideration of experimental error, there is no dramatic 20 difference in the replication of CV890 and CV802 in AFP<sup>+</sup> hepatoma cells. However, the growth curves of CV890 in AFP<sup>-</sup> cells showed clear attenuation. During a 5 day experiment, CV890 failed to replicate in AFP<sup>-</sup> cells including hepatoma cell (Change liver) and primary cells (nHLFC). From all the *in vitro* virus replication studies, it is clear that 25 replication of CV890 is under the tight control of AFP-TRE and this adenovirus variant has an excellent specificity profile of preferentially targeting AFP producing hepatocellular carcinoma cells.

#### In Vivo Specificity and Efficacy of CV890.

CV890 specificity was also evaluated in animals bearing prostate cancer LNCaP 30 xenografts. In this *in vivo* test, nude mice with prostate xenograft were intravenously injected with either CV890 or CV787, a prostate cancer specific adenovirus variant (Yu et al., 1999, *Cancer Research*, 59:4200-4203). Tumor volumes were documented and indicated that only CV787 had a significant antitumor efficacy in LNCaP xenografts, while tumors in the animals treated with CV890 grew, from 400 mm<sup>3</sup> to approximately 1200

mm<sup>3</sup> in six weeks, similar to the group treated with vehicle. This study indicates that CV890 does not attack LNCaP xenograft and keeps the good specificity profile under *in vivo* conditions.

To evaluate *in vivo* antitumor efficacy of CV890, different studies were carried out  
5 in the nude mouse model harboring human hepatoma xenografts. First, BALB/c nu/nu mice with HepG2 or Hep3B xenografts were established, animals were further challenged with single dose or multiple doses of CV890 into the tumor mass (intratumoral administration, IT) or via their tail vein (intravenous administration, IV). Tumor volume and the level of serum AFP were monitored weekly after the start of treatment, and hence  
10 the efficacy of the treatment was determined. The *in vitro* cytotoxicity study has demonstrated that CV890 has a better cytolytic effect than CV732. In order to further examine their antitumor activity, we first conducted animal study to compare CV890 to CV732. Animals harboring 300 mm<sup>3</sup> Hep3B xenograft were grouped (n=6) and injected with vehicle alone (control group), CV890 (1x10<sup>11</sup> particles/dose, CV890 group), or  
15 CV732 (1x10<sup>11</sup> particles/dose, CV732 group). The Hep3B xenograft is a very aggressive tumor model and tumors grow very fast. Most animals can not survive long because of excessive tumor burden. During a six week study, single intravenous administration of CV890 have shown significant tumor growth inhibition, whereas control mice had over 10  
fold tumor growth at week 5. In the group treated with CV732, single dose IV injection  
20 also reduced the tumor growth as compared to control group, however, it was much less effective compared to CV890. For example, the average tumor volume of the CV890 treated group dropped from 312 mm<sup>3</sup> to 219 mm<sup>3</sup>, while tumor volume increased from 308 mm<sup>3</sup> to 1542 mm<sup>3</sup> 5 weeks after treatment in control. Both control group and the CV732 group were terminated at week 5 because excessive tumor size. Previously, CV732 has  
25 been demonstrated to restrict the hepatoma tumor from growth after 5 doses of intravenous administration. Similar efficacy can be achieved with just a single intravenous administration of CV890, indicating that under *in vivo* conditions, CV890 has better efficacy than CV732 in hepatoma xenografts. In this experiment, 4 out of five CV890 treated mice were tumor free three weeks after treatment. However, in CV732 group,  
30 xenografts in two mice stopped growing but none of treated animals were tumor free through the six-week experiment. There was no tumor reduction in this group or the control group of animals. By statistical analysis, the differences in mean relative tumor volumes and serum AFP concentrations between CV890 treated and CV732 treated or

vehicle treated tumors are significant ( $p<0.01$ )). Taken together, these studies suggest that CV890 has a significant antitumor activity and its oncolytic efficacy is better than CV732, an adenovirus variant similar to AvE1a04I, in which the AFP TRE was applied to control E1A alone (Hallenback et al, 1999, Hum. Gene. Ther., 10:1721-1733).

5           Synergistic Antitumor Efficacy of CV890 in Combination with  
          Chemotherapeutic Agents

In this example, different chemotherapeutic agents were tested in combination with CV890 for their *in vitro* killing effect in Hep3B or HepG2 cells. Drug concentrations were optimized to the extent that they would not generate extensive cytotoxic effect on their own. Under such conditions, some agents had shown higher cell killing effect in combination with CV890. Among them, doxorubicin, a drug currently used in treatment of HCC showed synergistic cytotoxicity with CV890. In experiments using doxorubicin together with CV890 on Hep3B cells, doxorubicin at 10ng/ml did not generate cytotoxicity, whereas CV890 at an MOI of 0.01 (pfu/cell) only had about 35% of cell killed at day 9.

10           However, when both were applied together, 90% cells were killed 9 days after treatment. In order to determine the potential synergistic effect from the combination treatment, the MTT cell viability data were subjected to further statistical analysis. Figure 10 shows a representative  $IC_{50}$  isobogram of doxorubicin and CV890 on Hep3B cells at day 5. First, the dose-response curves of doxorubicin alone or CV890 alone were made. Based on the original theory of Steel and Peckham (1993) and method by Aoe et al. (1999), three isoeffect curves (mode I and mode 2a, 2b) were constructed. From this isobogram, several data points were in the synergy or additive area, indicating that combination of CV890 and doxorubicin provides synergistic effect on killing of Hep3B cells.

15           

20           

25           

Although CV890 alone has good antitumor activity, we applied combination therapy with doxorubicin for *in vivo* evaluation of synergy. Animals harboring 300 mm<sup>3</sup> Hep3B xenografts were grouped ( $n=6$ ) and injected with vehicle alone (control group), CV890 alone ( $1 \times 10^{11}$  particles/dose, CV890 group), doxorubicin alone (10mg/kg, doxorubicin group), or CV890 in combination with doxorubicin (combination group). Figure 11 shows weekly change of the relative tumor size normalized to 100% at day 1. In this experiment, by week six, all animals in the control group had excessive tumor which has increased by 700% of baseline, whereas in CV890 group and combination group, animals had either tumor free or tumor reduction. Of the eight Hep3B xenografts, treated with CV890, three animals (37.5%) had no palpable tumor at week 5, another three animals

30

had tumor regressed by more than 60%. In combination group, four out of eight animals were tumor free from week 5, another four animals had tumor reduction about 90%. All the animals in the CV890 and combination group were alive and tumor was suppressed even ten weeks following treatment whereas the control animals were sacrificed for 5 excessive tumor burden after week 6. Furthermore, CV890 also caused a drop in the serum AFP concentration in these mice. Statistical analysis shows that differences in mean relative tumor volumes and serum AFP concentrations between CV890 and vehicle treated group or combination and doxorubicin treated group are significant at different times ( $p<0.005$ ).

10 The strong efficacy in the combination treatment shows that single IV injection of CV890 in combination of doxorubicin can eradicate aggressive Hep3B xenografts in most of the animals.

15

Table 7. AFP production in different tumor cellsAFP

CELLS	(ng/ $10^6$ cells/10days)	
Hep3B	2645	
HepG2	3140	High
HuH7	4585	
SNU449	60	
PLC/PRF/5	600	Low
Chang	0	
SK-Hep1	0	
HBL100	0	None
PA-1	0	
LoVo	0	

## TABLE 1 IRES SEQUENCES

SEQ ID NO:1 A 519 base pair IRES obtainable from encephelomyocarditis virus (EMCV).

1	<b>GAC<u>TCGACTAATTCCGGTTATTTCCACCATATTGCCGTCTTGGCAA</u></b>
5	<i>Sall</i>
51	TGTGAGGGCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCCCTAGGG
101	GTCTTCCCCTCTGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAG
151	GAAGCAGTTCTCTGGAAGCTTCTGAAGACAAACACGTCTGTAGCGAC
201	CCTTGAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGCC
10	251 AAAAGCCACGTGTATAAGATAACACCTGCAAAGCGGCACAACCCCAGTGC
301	CACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAG
351	CGTATTCAACAAGGGGCTGAAGGATGCCAGAAGGTACCCCATTGTATGG
401	GATCTGATCTGGGCCTCGGTGCACATGCTTACATGTGTTAGTCGAGG
451	TTAAAAAAACGTCTAGGCCCCCGAACACGGGACGTGGTTTCCTTGA
15	<i>Sall</i>
501	<b>AAAACACGAT<u>TCGACGTC</u></b>

SEQ ID NO:2 An IRES obtainable from vascular endothelial growth factor (VEGF).

20	1 ACGT <u>AGTCGACAGCGCAGAGGCTTGGGCAGCCGAGCGGCAGCCAGGCC</u>
	<i>Sall</i>
51	CGGCCCAGGGCCTCGGTTCCAGAAGGGAGAGGAGGCCAGGCGCGCAA
101	GAGAGCGGGCTGCCTCGCAGTCCAGGCCAGAGGGAGCGCGAGCCGCGC
151	CGGCCCCGGACGGCCTCCGAAACCAT <u>GGTCGACACGTA</u>
25	<i>Sall</i>

SEQ ID NO:3 A 5'UTR region of HCV.

1	GCCAGCCCCCTGATGGGGCGACACTCCGCCATGAATCACTCCCTGTGAGGAACACTTG
61	TCTTCACGCAAAAGCGTCTAGCCATGGCCTAGTATGAGTGTGGTGCAGCCTCCAGGAC
30	121 CCCCCCTCCGGGAGAGCCATAGTGGCTGCCAACGGTGAGTACACCGGAATTGCCAG
181	GACGACCGGGCCTTCTGGATTAACCGCTCAATGCCCTGGAGATTGGCGTGCCCCC
241	GCAAGACTGCTAGCCGAGTAGTGTGGTCCGAAAGGCCTGTGGTACTGCCCTGATAGG
301	GTGCTTGCGAGTGCCCCGGAGGTCTCGTAGACCGTGCACC (341)

35 SEQ ID NO:4A 5'UTR region of BiP SEQ ID NO:4

1	CCCGGGGTCACTCCTGCTGGACCTACTCCGACCCCTAGGCCGGAGTGAAGGCGGGACT
61	TGTGCGGTTACCAGCGGAAATGCCCTGGGTCAAGTCGAGGAGAGATAGACAGCTGC
121	TGAACCAATGGGACCAGCGGATGGGGCGGATGTTATCTACCATTGGTGAACGTTAGAAC

181 GAATAGCAGCCAATGAATCAGCTGGGGGGCGGAGCAGTGACGTTATTGCGGAGGGGC  
241 CGCTTCGAATCGCGGCGGCCAGCTGGTGGCCTGGCCAATGAACGGCCTCCAACGAGC  
301 AGGGCCTTCACCAATCGCGGCCCTCACGACGGGCTGGGGAGGGTATATAAGCCGAGT  
361 AGGCGACGGTGAGGTCGACGCCGCCAAGACAGCACAGACAGATTGACCTATTGGGTG  
5 421 TTCGCGAGTGTGAGAGGGAAGCGCCGCCAGCTGTATTCTAGACCTGCCCTCGCCTGGT  
481 TCGTGGCGCCTTGTGACCCGGGCCCTGCCGCTGCAAGTCGAAATTGCCTGTGCTCC  
541 TGTGCTACGGCCTGTGGACTGCCTGCTGCCAACTGGCTGGCAAGATG (595)

SEQ ID NO:5 A 5'UTR of PDGF SEQ ID NO:5

10 1 GTTTGCACCTCTCCCTGCCCGGGTGCTCGAGCTGCCGTTGCAAAGCCAACTTGGAAAAA  
61 61 GTTTTTGGGGGAGACTTGGCCTTGAGGTGCCAGCTCCGCGCTTCCGATTTGGGG  
121 121 CTTTCCAGAAAATGTTGCAAAAAAGCTAAGCCGGGGCAGAGGAAAACGCTGTAGCCG  
181 181 GCGAGTGAAAGACGAACCATCGACTGCCGTGTTCTTCCCTTGGAGGTTGGAGTCCC  
241 241 TGGGCGCCCCACACCCCTAGACGCCCTGGCTGGTCGCGACGCAGCCCCCGGCCGTGG  
15 301 301 ATGCTGCACTCGGCTCGGATCCGCCAGGTAGCCGCCCTGGACCCAGGTCTGCC  
361 361 CAGGTCCTCCCCCTGCCCGGAGCGACGGAGCCGGGGCCGGCGGCCGGGGCA  
421 421 TGCGGGTGAGCCGCGGCTGCAGAGGCCTGAGCGCCTGATGCCGGACCTGAGCCGAGC  
481 481 CCACCCCCCTCCCCAGCCCCCACCCTGGCCGCGGGGGCGGCCGCTCGATCTACCGTC  
541 541 CGGGGCCCCCGCGGGCCGGGGCCGGAGTCGGCATG (575)

TABLE 2 LITERATURE REFERENCES FOR IRES

IRES Host	Example	Reference
Picornavirus	HAV	Glass et al., 1993. Virol 193:842-852
	EMCV	Jang & Wimmer, 1990. Gene Dev 4:1560-1572
	Poliovirus	Borman et al., 1994. EMBO J 13:3149-3157
HCV and pestivirus	HCV	Tsukiyama-Kohara et al., 1992. J Virol 66:1476-1483
	BVDV	Frolov I et al., 1998. RNA. 4:1418-1435
Leishmania virus	LRV-1	Maga et al., 1995. Mol Cell Biol 15:4884-4889
Retroviruses	MoMLV VL30 (Harvey murine sarcoma virus)	Torrent et al., 1996. Hum Gene Ther 7:603-612
	REV	Lopez-Lastra et al., 1997. Hum Gene Ther 8:1855-1865
Eukaryotic mRNA	BiP	Macejak & Sarnow, 1991. Nature 353:90-94
	antennapedia mRNA	Oh et al., 1992. Gene & Dev 6:1643-1653
	FGF-2	Vagner et al., 1995. Mol Cell Biol 15:35-44
	PDGF-B	Bernstein et al., 1997. J Biol Chem 272:9356-9362
	IGFII	Teerink et al., 1995. Biochim Biophys Acta 1264:403-408
	eIF4G	Gan & Rhoads, 1996. J Biol Chem 271:623-626
	VEGF	Stein et al., 1998. Mol Cell Biol 18:3112-3119; Huez et al., 1998. Mol Cell Biol 18:6178-6190

TABLE 3 TRE SEQUENCES

Nucleotide sequence of a human uroplakin II 5' flanking region. Position +1 (the translational start site) is denoted with an asterisk. SEQ ID NO:6 (number 1 of SEQ ID NO:6 corresponds to position -2239 with respect to the translational start site).

1	TCGATAGGTA	CCCACTATAG	GGCACCGGTG	GTCGACGGCC	CGGGCTGGTC	
5	51	TGGCAACTTC	AAGTGTGGGC	CTTTCAGACC	GGCATCATCA	GTGTTACGGG
10	101	GAAGTCACTA	GGAATGCAGA	ATTGATTGAG	CACGGTGGCT	CACACCTGTA
15	151	ATCCCAACAC	TCTGGGAGGC	CAAGGCAGGT	GGATCACTTG	TGGTCAGGAG
20	201	TTTGAGACCA	GCCTGGCCAA	CATGGTGAAA	CCTCATCTCT	ACTAAAAATA
25	251	CAAAAATTAG	CTGGGAATGG	TGGCACATGC	CTATAATCCC	AGTTACTCAG
30	301	GAGGCTGAGG	CAGGAGAACATC	ATTTGAACCT	GGGAGGCAGA	GGTTGCAGTG
35	351	AGCCGAGATC	ACGCCACTGC	ACTCCAGCCT	GGGTGACACA	GCGAGACTCT
40	401	GTCTCAAAAA	AAAAAAAATG	CAGAATTTCA	GGCTTCACCC	CAGACCCACT
45	451	GCATGACTGC	ATGAGAAGCT	GCATCTTAAC	AAGATCCCTG	GTAATTCTATA
50	501	CGCATATTAA	ATTGGGAGAT	GCACTGGCGT	AAGACCCCTCC	TACTCTCTGC
55	551	TTAGGCCCAT	GAGTTCTTCC	TTTACTGTCA	TTCTCCACTC	ACCCCAAAC
60	601	TTGAGCCTAC	CCTTCCCACC	TTGGCGGTAA	GGACACAAACC	TCCCTCACAT
65	651	TCCTACCAGG	ACCCTAACGCT	TCCCTGGGAC	TGAGGAAGAT	AGAATAGTTC
70	701	GTTGGAGCAAA	CAGATATACA	GCAACAGTCT	CTGTACAGCT	CTCAGGCTTC
75	751	TGGAAGTTCT	ACAGCCTCTC	CCGACAAAGT	ATTCCACTTT	CCACAAGTAA
80	801	CTCTATGTGT	CTGAGTCTCA	GTTCACACTT	TTCTCTCTCT	CTCTCTCTCT

851	CAACTTTCTG	AGACAGAGTT	TCACTTAGTC	GCCCAGGCTG	GAGTGCAGGG
901	GCACAATCTC	GGCTCACTGC	AACCTCCACC	TCCTGGGTTTC	AAGTGTTC
951	CCTGTCTCAG	CCTCCCGAGT	AGCTGGGATT	ACAGGCACAC	ACCACCGCGT
1001	TAGTTTTGT	ATTTTGGTA	GAGATGGTGT	TTCGCCATAT	TGGCCAGGCT
1051	GATCTCGAAC	TCCTGACCTC	AGGTGATCCG	CCCACCTCGG	CCTCCCAAAG
1101	TGCTGGGATT	ACAGGCATGA	GCCACCACGC	CCGGCTGATC	TCTTTCTAT
1151	TTTAATAGAG	ATCAAACCTCT	CTGTGTTGCC	TAGGCTGGTC	TTGAACTCCT
1201	GGCCTCGAGT	GATCCTCCCA	CCTTGGCCTC	CCAAAGTGT	GAGATTACAG
1251	GCATGAGCCA	CTGTGCCTGG	CCTCAGTTCT	ACTACAAAAG	GAAGCCAGTA
1301	CCAGCTACCA	CCCAGGGTGG	CTGTAGGGCT	ACAATGGAGC	ACACAGAAC
1351	CCTACCCAGG	GCCCCGAAGA	AGCCCCGACT	CCTCTCCCCT	CCCTCTGCC
1401	AGAACTCCTC	CGCTTCTTTC	TGATGTAGCC	CAGGGCCGGA	GGAGGCAGTC
1451	AGGGAAGTTC	TGTCTCTTTT	TCATGTTATC	TTACGAGGTC	TCTTTCTCC
1501	ATTCTCAGTC	CAACAAATGG	TTGCTGCCA	AGGCTGACTG	TGCCCAACCC
1551	CAACCCCTGC	TGGCCAGGGT	CAATGTCTGT	CTCTCTGGTC	TCTCCAGAAG
1601	TCTTCCATGG	CCACCTTCGT	CCCCACCCCTC	CAGAGGAATC	TGAAACCGCA
1651	TGTGCTCCCT	GGCCCCCACA	GCCCCTGCCT	CTCCCAGAGC	AGCAGTACCT

1701	AAGCCTCAGT	GCACCTCAAG	AATTGAAACC	CTCAGTCTGC	TGCCCTCCCC
1750					
1751	CACCAGAATG	TTTCTCTCCC	ATTCTTACCC	ACTCAAGGCC	CTTTCAGTAG
1800					
1801	CCCCTGGAG	TATTCTCTTC	CTACATATCA	GGGCAACTTC	CAAACATCATC
1850					
1851	ACCCTTCTGA	GGGGTGGGGG	AAAGACCCCC	ACCACATCGG	GGGAGCAGTC
1900					
1901	CTCCAAGGAC	TGGCCAGTCT	CCAGATGCC	GTGCACACAG	GAACACTGCC
1950					
1951	TTATGCACGG	GAGTCCCAGA	AGAAGGGGTG	ATTCTTTCC	CCACCTTAGT
2000					
2001	TACACCATCA	AGACCCAGCC	AGGGCATCCC	CCCTCCTGGC	CTGAGGGCCA
2050					
2051	GCTCCCCATC	CTGAAAAAAC	TGTCTGCTCT	CCCCACCCCT	TTGAGGCTAT
2100					
2101	AGGGCCCAAG	GGGCAGGTTG	GACTGGATT	CCCTCCAGCC	CCTCCCGCCC
2150					
2151	CCAGGACAAA	ATCAGCCACC	CCAGGGGCAG	GGCCTCACTT	GCCTCAGGAA
2200					
2201	CCCCAGCCTG	CCAGCACCTA	TTCCACCTCC	CAGCCCAGCA	
2239					

Nucleotide sequence of a mouse uroplakin II 5' flanking region. The translational start site is denoted with an asterisk. SEQ ID NO:7 (number 1 of SEQ ID NO:7 corresponds to position -3592 with respect to the translational start site).

1	CTCGAGGATCTGGCCCTTTCTGCATCCTGTCCTAAATCATTTCAT	50
51	ATCTTGCTAGACCTCAGTTGAGAGAAACGAACCTCTCATTTCAAGTT	100
101	GAAAAAAAAAGAGGTTCAAAGTGGCTCACTCAAAGTTACAAGCCAACAC	150
151	TCACCACTACGAGTACAATGCCACCATTAGTGCTGGCATGCCAGGAG	200

ACAGGCATGCATATTATTCTAGATGACTGGGAGGCAGAGGGGTGGCCTAG  
201 250

5 TGAGGTCAGACTGTGGACAGATCAGGCAGATGTGGTTCTGATCCAAATT  
251 300

CCTCAGGCCGCAGAACTACTGTGGTTCAAGAAGGGGACAAAAGGACTGCA  
301 350

10 GTCCGGAACAGGAGGTCCATTGAGAGCTGACTGAGCAGAAGAGGAAAGT  
351 400

15 GAAGAACTTCTGGGGCAAGAGCTTACCTACTTACAGCTTGTTGTCTT  
401 450

CTTTACTCCAGGGCGTCCTGGTACTCAGTAAATGTCTGTTGGCTTGAG  
451 500

20 GAACATATGTGTAAGGAGGAAGGAGAGGAACTTGAGGGAGTTAAGACTC  
501 550

AAGAATCAATCAAGGAGAGGACAGCAGAGAAGACAGGGTTGGAGAGAG  
551 600

25 ACTCCAGACATTGGCCCTGGTCCCTTCTTGGCCACTGTGAAACCCCTCCA  
601 650

GAGGAACTGAGTGCTGTGGCTTAAATGATCTCAGCACTGTCACTGAAGC  
651 700

30 GCTCTGCTCAAAGAGTTATCCTCTTGCTCCTGTGCCGGGGCTCCCCCTC  
701 750

CTCTCAGCTCCAAACCCCTCTCAGCCACTGTGATGGCATAATTAGATGC  
751 800

GAGAGCTCAGACCGTCAGGTCTGCTCCAGGAACCACCCATTTCACCAAC  
801 850

40 CCCAGAGAAAGGTCTAGTGGAAAAGTGGGGCCACTGAAGGGCTGATGG  
851 900

GGTTCTGTCCTTCCCCATGCTGGTGGACTTAAAGTCTGCGATGTGTG  
900 950

45 TAGGGGGTAGAACACAGAACCTGGGGCTCCGGCTGGAGCAGGAGG  
951 1000

AACTCTACCAAGACGATCTCAAATTACTGTGCAATGGACGATCAGGAA  
1001 1050

ACTGGTTCAAGATGTAGCTCTGATACAGTGGGTCTGAGGTAAAACCGAA  
1051 1100

55 ACTTAATTCTTCAAAATTAAAGTTGCATTATTATTTATATGTGT  
1101 1150

GCCCATATGTGTGCCACAGTGTCTATGTGGAGGTAGAGGGCAAGTTGTG  
 1151 1200

5 GGCATTGGCTCTCTCCTTCATAATGTGGCTCTGGGGACCAAAATGTCA  
 1201 1250

GGCATGGTGGCAAGAGCTTTACCTGTTGAGCCATCTCATGGTTCGTAA  
 1251 1300

10 AACTCCTATGACGCTTACAGGTAACGCAGAGACACAGACTCACATTGG  
 1301 1350

15 AGTTAGCAGATGCTGTATTGGTGTAAACACTCATACACAGACACACACAC  
 1351 1400

ATACTCATACACACACACACACTTATCACATGCACACACATACTCGTA  
 1401 1450

20 TACACACAGACACACACACATGCACTCTCACATTACATATTACATACACA  
 1451 1500

TCCACACACACACTCATCCACACACAGACACACATACTCATCCACACA  
 1501 1550

25 CACACACACACATACTCATACACACACAGACACACATACTCATACACA  
 1551 1600

30 CACACAGACACACACATATAATCATAACATACACAGACACACTCATACATG  
 1601 1650

TGCACACACACACTCATCCACACACACACTCATACACACACACACTCA  
 1651 1700

35 TACACACACACACTCATACACACACACACACGAGGTTTCAGCTCCTGGCTTTG  
 1701 1750

TTGGGTGGAGACTGGAAGTGATTCTGTTTCAGCTCCTGGCTTTG  
 1751 1800

40 TCCCTTAGATGAGATCTCCTCCTCACTTACACACAGAAAGATCACACA  
 1801 1850

45 CGAGGGAGAACTGGCGGTGCGGAAGAGGGCTACACGGTAGGGTGTCAAGGG  
 1851 1900

TCAGGAGATCTCCTGGCAAGTCTCAAACCTCCACATAGCACAGTGTAA  
 1901 1950

50 CGTGAGGATTTAGGAGGAATCAGGAAGAGGGATTGGTTACTGCAGAGCAG  
 1951 2000

ACCATATAGGTCCACTCCTAACGCCATTGAAATTAGAAGTGAGACAGT  
 2001 2050

55 GTGGGATAAAAAGAGCAGATCTCTGGTCACATTAAAGGGATATGAGG

2051 3000  
GTCCTGTGCCTTAAGCCTCCCCTCCATCTCCCTCCAATCCCCCTCACCTC  
2101 2150  
5 CCCACCCCTAACCCCTCCCCAGGTTCTGGAGGAGCAGAGTTGCGTCTTCTC  
2151 2200  
CCTGCCCTGCCGAGCTGCTCACTGGCTGCTCTAGAGGCTGTGCTTGC  
10 2201 2250  
TCTCCATGGAAACCATTAGTTGCTAAGCAACTGGAGCATCTGTGCTG  
2251 2300  
15 AGCTCAGGTCCATCGAGTTCACCTAGCTGAGACACCCACGCCCTGCAG  
2301 2350  
CCACTTGCAGTGACAAGCCTGAGTCTCAGGTTCTGCATCTATAAAAACG  
20 2351 2400  
20 AGTAGCCTTCAGGAGGGCATGCAGAGCCCCCTGGCCAGCGTCTAGAGGA  
2401 2450  
25 GAGGTGACTGAGTGGGCCATGTCACTCGTCCATGGCTGGAGAACCTCCA  
2451 2500  
TCAGTCTCCCAGTTAGCCTGGGCAGGAGAGAACAGAGGAGCTGTGGCT  
2501 2550  
30 GCTGATTGGATGATTACGTACCCAATCTGTTGCCAGGCATCGAACCC  
2551 2600  
CAGAGCGACCTGCACACATGCCACCGCTGCCCGCCCTCCACCTCCTCTG  
35 2601 2650  
35 CTCCTGGTTACAGGATTGTTGTCTTGAAAGGGTTTGTTGTTGCTACTT  
2651 2700  
40 TTTGCTTGTGTTTTCTTTAACATAAGGTTCTCTGTGTAGCCCTAG  
2701 2750  
CTGTCCTGGAACTCACCTGTAGACCAGGCTGGCCTCAAACTCAGAAATC  
2751 2800  
45 CACCTTCCTCCAAAGTGCTGGATTAAAGGCATTGCAACCATGCCAGC  
2801 2850  
CCCCGGTCTGTTCTAACAGGTTCTGCTTACTCGCTACCGTTGCA  
50 2851 2900  
50 CAACCGCTTGTCCAAGTCTGTTGTATCTACTCCACCGCCACTAGC  
2901 2950  
55 CTTGCTGGACTGGACCTACGTTACCTGGAAGCCTCACTAACTCCCTT  
2951 3000

	GTCTCCACCTCTGGAGAAATCTGAAGGCTCACACTGATACCCCTCCGCTT	3001	3050
5	CTCCCAGAGTCGCAGTTCTTAGGCCTCAGTTAAATACCAATTGGATC	3051	3100
	TCAGGCTCTGCTATCCCCACCCCTACCTAACCAACCCCTCCTCTCCCAC	3101	3150
10	CTTACTAGCAAAGCCCTTCAACCCTGGGGCTTCTACACCTACAC	3151	3200
	ACCAGGGCAATTAGAACTCATGGCTCTCCTAGAAAACGCCTACCTCC	3201	3250
15	TGGAGACTGACCCCTACAGTCCAGGAGGCAGACACTCAGACAGAGGAAC	3251	3300
20	TCTGTCCTTCAGTCGCCGGAGTCCAGAAAGAGCCATACTCCCCTGCAGA	3301	3350
	GCTAACTAAGCTGCCAGGACCCAGCCAGAGCATCCCCCTTAGCCGAGGG	3351	3400
25	CCAGCTCCCCAGAATGAAAAACCTGTCTGGGGCCCTCCCTGAGGCTACA	3401	3450
	GTCGCCAAGGGCAAGTGGACTGGATTCCAGCAGCCCTCCACTCCG	3451	3500
30	AGACAAAATCAGCTACCCGGGCAGGCCTCATTGGCCCCAGGAAACCC	3501	3550
	AGCCTGTCAGCACCTGTTCCAGGATCCAGTCCAGCGCAGTA	3551	3592

## AFP-TRE. SEQ ID NO:8.

40	1	GCATTGCTGTAACTCTGTACTTAGGACTAAACTTGAGCAATAACACACATAGATTGAG	
	61	GATTGTTGCTGTTAGCATACAAACTCTGGTCAAAGCTCCTCTTATTGCTTGTCTTGG	
45	121	AAAATTGCTGTTCTCATGGTTCTCTTTCACTGCTATCTATTCTCAACCACTCA	
	181	CATGGCTACAATAACTGTCTGCAAGCTTATGATTCCAAATATCTATCTAGCCTCAAT	
	241	CTTGTCCAGAAGATAAAAGTAGTATTCAAATGCACATCAACGTCTCCACTGGAGGGC	
	301	TTAAAGACGTTCAACATACAAACCGGGAGTTTGCCTGGAATGTTCTAAAATGTGT	
50	361	CCTGTAGCACATAGGGCTCTTGTCTTAAATCTAATTACTTTAGCCAGTGCTCA	
	421	TCCCACCTATGGGGAGATGAGAGTGAAAAGGGAGCCTGATTAATAATTACACTAAGTCAA	

481 TAGGCATAGAGCCAGGACTGTTGGTAAACTGGTCACTTATCTTAAACTAAATATTC  
 541 CAAAACGTAAACATGTACTTAGTTACTAAGTCTTGACTTTATCTCATTCAACACTCAG  
 5 601 CTTTATCCAGGCCACTTATGAGCTCTGTGTCCTTGAACATAAAATACAAATAACCGCTAT  
 661 GCTGTTAATTATTGGCAAATGTCCCATTTCACCTAACGAAATACCAAAAGTAACAGA  
 721 TATACCAACAAAAGGTTACTAGTTAACAGGCATTGCCTGAAAAGAGTATAAAAGAATTTC  
 10 781 AGCATGATTTCCATATTGTGCTTCCACCACTGCCAATAACA (822)

15

Probasin-TRE SEQ ID NO:9

20 <sup>-426</sup>  
 5' -AAGCTTCACAAAGTGCATTTAGCCTCTCCAGTATTGCTGATGAATCCACAGT  
 TCAGGTTCAATGGCGTTCAAAACTTGATCAAAATGACCAGACTTTATTTA  
 CACCAACATCTATCTGATTGGAGGAATGGATAATAGTCATCATGTTAAACAT  
 25 CTACCATTCCAGTTAAGAAAATATGATAGCATTTGTTCTTAGTCTTTCTTA  
 ARE-1  
 ATAGGGACATAAAGCCCACAAATAAAATATGCCTGAGAAATGGGACAGGC  
 30 ATTGGGCATTGTCCATGCCTAGTAAAGTACTCCAAGAACCTATTGTATACTA  
 ARE-2  
 GATGACACAAATGTCAATGTCTGTACAACGTCCAACTGGGATGCAAGACAC  
 TGCCCATGCCAATCATCCTGAAAAGCAGCTATAAAAGCAGGAAGCTACTCT  
 35 CAAT box TATAA box  
 +1 +28  
 GCACCTTGTCAAGTAGGTCCAGATAACCTACAG-3'  
 Transcription site  
 40

Tyrosinase-TRE. SEQ ID NO:10

45 PinAl end -1956  
 1 CCGG<sup>0</sup> AAAATGATAAGTTGAATTCTGTCTCGAGAACATAGAAAAGAA  
 51 TTATGAAATGCCAACATGTGGTTACAAGTAATGCAGACCCAAGGCTCCCC  
 101 AGGGACAAGAAGTCTTGTGTTAATCTTGTGGCTCTGAAAGAAAGAGAG  
 151 AGAGAAAAGATTAAGCCTCCTGTGGAGATCATGTGATGACTTCCTGATT  
 201 CCAGCCAGAGCGAGCATTCATGGAAACTTCTCTCCCTTTC<sup>-1716</sup> CTCGAG  
 50 -23<sup>1</sup> 251 ATTACTAACCTTATTGTTAATATTCTAACATAAGAATTAAACTATTAAT  
 301 GGTGAATAGAGTTTCACTTAACATAGGCCTATCCCACGGTGGGATA  
 351 CGAGCCAATTGAAAGAAAAGTCAGTCATGTGCTTCAGAGGATGAA + 65

401 GCTTAAGATAAAAGACTAAAAGTGTGATGCTGGAGGTGGGAGTGGTATT  
451 ATATAGGTCTCAGCCAAGACATGTGATAATCACTGTAGTAGCTGGAA  
501 AGAGAAACTGTGACTCCAATTAGCCAGTTCCCTGCAGACCTTGTGA

PinAl end

## 5 Human glandular kallikrein-TRE SEQ ID NO:11

10 gaattcagaa ataggggaag gttgaggaag gacactgaac tcaaaggga tacagtgatt 60  
ggtttatttg tcttctcttc acaacattgg tgctggagga attcccaccc tgaggttatg 120  
aagatgtctg aacacccaac acatagcact ggagatatga gctcgacaag agtttctcag 180  
ccacagagat tcacagccta gggcaggagg acactgtacg ccaggcagaa tgacatggga 240  
15 attgcgctca cgattggctt gaagaagcaa ggactgtggg aggtgggctt tgttagtaaca 300  
agagggcagg gtgaactctg attcccatgg gggaatgtga tggtcctgtt acaaattttt 360  
caagctggca gggaaataaaa cccattacgg tgaggacctg tggagggcgg ctgccccaaac 420  
20 tgataaagga aatagccagg tgggggcctt tcccattgtt ggggggacat atctggcaat 480  
agaagccttt gagacccttt agggtacaag tactgaggca gcaaataaaa tgaaatctta 540  
25 ttttcaact ttatactgca tgggtgtgaa gatataatttg tttctgtaca ggggtgagg 600  
gaaaggaggg. gaggaggaaa gttcctgcag gtctggtttgc ttttgtgat ccagggggtc 660  
ttggaactat ttaaattttaa ttaaattttaa acaagcgact gttttaaattt aaattttaaattt 720  
30 aaattttaaattt ttactttattt ttatcttaag ttctggcttca catgtgcagg acgtgcagct 780  
ttgttacata ggttaaacgtg tgccatggtg gtttgctgttca cctatcaacc catcacctag 840  
35 gtattaagcc cagcatgcat tagctgtttt tcctgacgct ctccctctcc ctgactcccc 900  
caacaggccc cagtgtgtgt tggcccttc cctgtgtcca tgggttctca ttgttcaagct 960  
ccccacttata agtgagaaca tgggtgtttt ggtttctgt ttctgtgttca gtttgctgag 1020  
40 gataatggct tccacacttca tccatgttcc tgcaaaggac gtgatcttac tcttttttat 1080  
ggttgcatac aaattgtttt tacaatcca attgatattt tatttaattt caagttaatc 1140  
45 taatttagcat actagaagag attacagaag atattaggtt cattgaatga gggaaatataat 1200  
aaaataggac gaaggtgaaa tatttaggtt gaaaagtata atagttgaaa gaagtaaaaa 1260  
aaaatatgca tgtagtagcag aatgtttttt aggtgaagaa cgtaatagtg actttttttaga 1320  
50 ccagattgaa ggacagagac agaaaaattt taaggaattt ctaaaccatg tgagtgttag 1380  
aagtacagtc aataacatta aagcctcagg aggagaaaag aataggaaag gaggaaatataat 1440  
55 gtgaataat agtaqagaca tgtttgatgg attttttttt atttggaaaga cctcacatca 1500

aaggattcat accgtgccat tgaagaggaa gatggaaaag ccaagaagcc agatgaaaagt 1560  
tagaaatatt attggcaaag cttaaatgtt aaaagtccct aagagaaaagg atggcagaaa 1620  
5 tattggcgaa aaagaatgca gaacctagaa tataaattca tcccaacagt ttggtagtgt 1680  
gcagctgttag cctttcttag ataatacact attgtcatac atcgcttaag cgagtgtaaa 1740  
atggtctcct cactttattt atttatataat ttattnatgtt ttgagatgga gcctcgctct 1800  
10 gtctcctagg ctggagtgcata atagtgcgtt accactca gcaacctctg cctcctctgt 1860  
tcaagtgatt ttcttacctc agcctccca gtagctggga ttacagggtgc gtgccaccac 1920  
15 acccggtctaa tttttgtatt tttttagag acggggttt gccatgttgg ccaggctgg 1980  
cttgaactcc tgacatcagg tgatccacct gccttggcct cctaaagtgc tggattaca 2040  
20 ggcatgagcc accgtgccc accactttat ttattnattna ttttatttt taaatttcag 2100  
cttctattna aaatacaggg ggcacatata taggattgtt acatgggtat attgaactca 2160  
ggttagtgcata atactaccca acaggttaggt tttcaacccca ctccccctct tttcctcccc 2220  
25 attctagtag tgtgcagtgt ctattgttct catgtttagt tctatgtgtc ctccagggtt 2280  
agctcccacc tgtaagttag aacgtgtggt attttagttt ctgtccctgt gtaattcac 2340  
ttaggattat ggcttccagc tccattcata ttgctgtaaa ggatatgatt cattttcat 2400  
30 ggcatgca gattccatat tgcgtataga tcacatttc tttcttttt tttttgaga 2460  
cgagtccttgc tttgctgccc taggctggag tgcagtagca cgatctcggc tcaactgcaag 2520  
35 cttcacctcc ggggttcacg tcattttct gtctcagctt cccaaatgc tggactaca 2580  
ggcgcccgcc accacgtccg gctaattttt ttgtgtgtt ttagtagaga tgggggtttc 2640  
40 actgtgttag ccaggatggt ctgtatctcc tgaccttgcgt gtccacactgc ctccgtctcc 2700  
caaagtgcgtt ggattacagg ggtgagccac tgcggccggc ccatatatac cacatttct 2760  
ttaaccaatc caccattgtt gggcaactag gtagattcca tggattccac agtttgcta 2820  
45 ttgtgtgcag tgtggcagta gacatataaa tgaatgtgtc tttttggat aatgatttgc 2880  
attcctttgg gtatacagtc attaataatggta gtgtgggtt gaacgggtggc tctgtttaaa 2940  
50 attcttttag aattttccaa actgtttgcc atagagagca aactaattta cattttccacg 3000  
aacagtataat aagcattccccc ttttctccac agctttgtca tcatggtttt ttttttctt 3060  
tattttaaaa aagaatataatgt ttgtgtttcc ccagggtaca tgtgcaggat gtgcagggtt 3120  
55 gttacatagg tagtaaacgt gagccatgggt ggtttgtgc acctgtcaac ccattacctg 3180  
ggtagatgac cctgcctgca ttagctttt tccctaatgc tctcaactact gccccaccc 3240  
caccctgaca gggcaaaacag acaacctaca gaatgggagg aaattttgc aatctattca 3300

5 tctgacaaag gtcaagaata tccagaatct acaaggaact taagcaaatt tttactttt 3360  
aataatagcc actctgactg gcgtgaaatg gtatctcatt gtggtttca tttgaatttc 3420  
tctgatgatc agtgacgatg agcattttt cataattgtt ggctgcttgt acgtctttg 3480  
agaagtgtct cttcatgcct tttggccact ttaatggat tatttttgc ttttagttt 3540  
10 aagttcccta tagattctgg atattagact tcttattgga tgcatagttt gtgaataactc 3600  
tcttccattc tgttaggttgt ctgttactc tattgatggc ttctttgct gtgccgaagc 3660  
15 atcttagttt aattagaaac cacctgccaa tttttgtttt tggtcaatt gctttgggg 3720  
acttagtcat aaactctttg ccaaggtctg ggtcaagaag agtatttcctt aggttttctt 3780  
ctagaatttt gaaagtctga atgtaaacat ttgcattttt aatgcacatc gagtttagttt 3840  
20 ttgtatatgt gaaaggtcta ctctcatttt ctttccctct ttctttctt ctttctttc 3900  
tttctttctt tctttctt tttctttctt tctttctt tttcttttgc ttctttctt 3960  
25 ttctttctt tctctttctt tctcttttc tttttttttt ttgatggagt attgctctgt 4020  
tgcccaggct gcagtgcagc ggcacgatct cggctcactg caacctctgc ctctgggtt 4080  
caactgattc tcctgcatca gccttccaag tagctggat tataggcgcc cgccaccacg 4140  
30 cccgactaat ttttgtattt ttagtagaga cggggttgtg ccatgttggc caggctggtt 4200  
tgaaactcct gacctcaaac gatctgcctg ccttggcctc ccaaagtgtt gggattacag 4260  
35 gtgtgagcca ctgtgcccag ccaagaatgt cattttctaa gaggtccaag aacctcaaga 4320  
tattttggga ctttgagaag agaggaattc atacaggtat tacaagcaca gcctaattggc 4380  
aaatctttgg catggcttgg cttcaagact ttaggctctt aaaagtcgaat tccaaaattt 4440  
40 tttataaaag ctccagctaa gctacctaa aaggggcctg tatggctgat cactcttctt 4500  
gctatactttt acacaaataa acaggccaaa tataatgagg ccaaaattta ttttgc当地 4560  
45 aaattggtcc tgctatgatt tactcttggt aagaacaggg aaaatagaga aaaatttaga 4620  
ttgcatctga ctttttttc tgaatttttataatgtgccta caatttgagc taaatcctga 4680  
attattttctt gtttgc当地aaa actctctaa gaagaacttg gtttcatgg tcttcgtgac 4740  
50 acatttatct ggctcttac tagaacagct ttcttgggg tgggtttctt gcttgggtgc 4800  
cttacagttt tactcttcaattattgtta tgggtatctc atagtttcc ttctttgag 4860  
aaaactgaag ccatggattt ctgaggacta gagatgactc aacagagctg gtgaatctcc 4920  
55 tcatatgcaa tccactgggc tcgatctgct tcaaattgtt gatgcactgc tgctaaagct 4980  
atacatttaa aaccctcaact aaaggatcag ggaccatcat ggaagaggag gaaacatgaa 5040

attgttaagag ccagattcg 5100  
agaaggtaaa gcaacctatc 5160  
5 gttcttagaa gactgacagt 5220  
atccccagtg ttcggaggtg 5280  
cttcttgc 5340  
10 tttttgtg gtgttacagt 5400  
tgcgtggccc ctcccctccc 5460  
gcttgcctt ctaccataag 5520  
15 tgcttcctgt acagcctgca 5580  
cagtctttag 5640  
tccaagttt 5700  
20 ggtcaaacaa ctttgatggc 5760  
cctccctat ggcataataag 5820  
ttgtctggct gccacactgag 5880  
ttctttctaa gaaactggat 5940  
30 ttgggttagg 5960  
atagatataa tccacataaa 6000  
aaatgtgtcc 6060  
35 tagagtgtgg 6120  
ggctgaagtc 6180  
40 catgttgcga 6240  
ggcatctaga 6300  
tcctggttct 6360  
45 ttcataataca 6420  
agaacctttc 6480  
50 catatgaata 6540  
ccttaaaaat 6600  
ttgccagtt 6660  
55 aagagtccct 6720  
agatttcaag 6780  
taactcaatg 6840

5 gaaaatctag caagggaga gttgcatggg gcaaagacaa ggagagctcc aagtgcagag 6900  
attcctgtt ttttctccca gtggtgtcat ggaaagcagt atcttctcca tacaatgtat 6960  
tgtgataata ttcagtgtat tgccaatcag ggaactcaac tgagccttga ttatattgga 7020  
gcttggttgc acagacatgt cgaccacctt catggctgaa cttagtact tagccccctcc 7080  
10 agacgtctac agctgatagg ctgtaaccca acattgtcac cataaatcac attgttagac 7140  
tatccagtgt ggcccaagct cccgtgtaaa cacaggcact ctaaacaggc aggatatttc 7200  
aaaagcttag agatgacctc ccaggagctg aatgcaaaga cctggcctct ttgggcaagg 7260  
15 agaattcctt accgcacact ctccttcaca gggttattgt gaggatcaa tgggtcatg 7320  
tgtgtgagac accagcacat gtctggctgt ggagagtgac ttctatgtgt gctaacattt 7380  
20 ctgagtgcta agaaagtatt aggcatggct ttcagcactc acagatgctc atctaattcct 7440  
cacaacatgg ctacagggtg ggcactacta gcctcatttg acagaggaaa ggactgtgga 7500  
25 taagaagggg gtgaccaata ggtcagagtc attctggatg caaggggctc cagaggacca 7560  
tgattagaca ttgtctgcag agaaattatg gctggatgtc tctgccccgg aaagggggat 7620  
gcactttcct tgaccctcta tctcagatct tgacttttag gttatctcag acttcctcta 7680  
30 tgataccagg agcccatcat aatctctctg tgtcctctcc ctttcctcag tcttactgcc 7740  
cactcttccc agctccatct ccagctggcc aggtgtagcc acagtaccta actctttgca 7800  
gagaactata aatgtgtatc ctacagggga gaaaaaaaaa aagaactctg aaagagctga 7860  
35 cattttaccg acttgcaaac acataagcta acctgccagt tttgtgctgg tagaactcat 7920  
gagactcctg ggtcagaggc aaaagatttt attacccaca gctaaggagg cagcatgaac 7980  
40 tttgtgttca catttgttca ctttgccttcc caattcatat gggatgatca gagcagttca 8040  
ggtggatgga cacaggggtt tgtggcaaag gtgagcaacc taggcttaga aatcctcaat 8100  
45 cttataagaa ggtactagca aacttgccttca gtctttgtat ctgacggaga tattatcttt 8160  
ataattgggt taaaacgaga cctactctgg aggaacatata tgtattttattt gtcctgaaca 8220  
gtaaacaaat ctgctgtaaa atagacgtt actttattat ctaaggcagt aagcaaacct 8280  
50 agatctgaag gcgataccat cttgcaaggc tatctgctgt acaaataatgc ttgaaaagat 8340  
ggtccagaaa agaaaacggt attattgcct ttgctcagaa gacacacaga aacataagag 8400  
55 aaccatggaa aattgtctcc caacactgtt caccacagac cttccactct tttctgcagg 8460  
acagtcttaa catcccatca ttagtgcgtc taccacatct ggcttcaccg tgcctaaacca 8520  
agatttcttag gtccagttcc ccaccatgtt tggcagtgcc ccactgccaa ccccagaata 8580

aggagtgct cagaattccg agggacatg ggtggggatc agaacttctg ggcttgagtg 8640  
cagagggggc ccatactcct tggcccgaa ggaggaagag gctggaggtg aatgtccttg 8700  
5 gaggggagga atgtgggttc tgaactctta aatccccaaag ggaggagact ggtaaggccc 8760  
cagcttccga ggtactgacg tggaaatggc ctgagaggc taagaatccc gtatccctgg 8820  
gaaggagggg ctgaaattgt gagggggtga gttcaggggg tttgttagct tgagactcct 8880  
10 tggtgggtcc ctggaaagca aggactggaa ccattggctc cagggtttgg tgtgaaggta 8940  
atggatctc ctgattctca aagggtcaga ggactgagag ttgcccatgc tttgatctt 9000  
15 ccatctactc cttactccac ttgagggtaa tcacctactc ttctagttcc acaagagtgc 9060  
gcctgcgcga gtataatctg cacatgtgcc atgtcccgag gcctggggca tcatccactc 9120  
20 atcattcago atctgcgcta tgcggcgcag gccggcgcca tgacgtcatg tagctgcgac 9180  
tatccctgca gcgcgcctct cccgtcacgt cccaaccatg gagctgtgga cgtgcgtccc 9240  
ctggtgatg tggcctgcgt ggtgccaggc cggggcctgg tgtccgataa agatcctaga 9300  
25 accacaggaa accaggactg aaagggtgcta gagaatggcc atatgtcgct gtccatgaaa 9360  
tctcaaggac ttctgggtgg agggcacagg agcctgaact tacgggtttg ccccagtcca 9420  
ctgtcctccc aagtgagtct cccagatacg aggactgtg ccagcatcag cttcatctgt 9480  
30 accacatctt gtaacaggaa ctacccagga ccctgatgaa caccatggtg tgtgcaggaa 9540  
gaggggggtga aggcatggac tcctgtgtgg tcagagccca gaggggggcca tgacgggtgg 9600  
35 ggaggaggct gtggactggc tcgagaagtg ggtatgtggtt gtgtttgatt tccttggcc 9660  
agataaagtg ctggatatacg cattgaaaaac ggagtatgaa gaccagtttag aatggagggt 9720  
40 caggttggag ttgagttaca gatggggtaa aattctgctt cggatgagtt tggggattgg 9780  
caatctaaag gtgggttggg atggcatggc tttgggatgg aaatagggtt gttttatgt 9840  
tggctggaa ggggtgtggg attgaattgg ggtatgaagta ggttttagtt tggagataga 9900  
45 atacatggag ctggctattg catgcagga tgtgcattag ttgggttga tccttaata 9960  
aaggaggcta ttagggttgt cttgaatttag attaagttgt gttgggttga tgggttggc 10020  
50 ttgtgggtga tgtgggttga ttgggtgtg ttaaattggt ttgggtcagg tttgggttga 10080  
ggttatcatg gggatgagga tatgcttggg acatggattc aggtgggtct cattcaagct 10140  
gaggcaaatt tccttcaga cggtcattcc agggAACGAG tgggtgtgtg gggaaatca 10200  
55 ggccactggc tgtgaatatc cctctatcct ggtcttgaat tgtgattatc tatgtccatt 10260  
ctgtctcatt cactgtactt ggaattgatc tggcattca gctggaaatg gggaaagatt 10320  
ttgtcaaatt cttgagacac agctgggtct ggatcagcgt aagccttcct tctggttta 10380

ttgaacagat gaaatcacat ttttttttc aaaatcacag aaatcttata gagttaacag 10440  
5 tggactctta taataagagt taacaccagg actcttattc ttgattctt tctgagacac 10500  
caaaatgaga tttctcaatg ccacccta at tctttttt tttttttt ttttgagac 10560  
acagtctggg tctttgctc tgtcaactca gctggagcgc agtggtgtga tcata gctca 10620  
10 ctgaaccctt gacccctgg acttaaggga tcctcctgct tcagcctcct gagtagatgg 10680  
ggctacaggt gcttgcacc acacctggct aattaaattt tttttttt tttgtagaga 10740  
aagggtctca ctttggccct ctggctgatc ttgaacttct gacttcaagt gattcttcag 10800  
15 ccttggactc ccaaagcaact gggattgctg gcatgagcca ctcaccgtgc ctggcttgca 10860  
gcttaatctt ggagtgtata aacctggctc ctgatagcta gacatttcag tgagaaggag 10920  
20 gcattggatt ttgcatgagg acaattctga cctaggaggg caggtcaaca ggaatcccc 10980  
ctgtacctgt acgttgtaca ggcattggaga atgaggagtg aggaggccgt accggAACCC 11040  
catattgttt agtggacatt ggattttgaa ataataggga acttggctcg ggagagtcat 11100  
25 atttctggat tggacaatat gtggtatcac aaggtttat gatgagggag aaatgtatgt 11160  
ggggAACCAT tttctgagtg tggaaagtgc a agaatcagag agtagctgaa tgccaacgct 11220  
30 tctatttcag gaacatggta agttggaggt ccagctctcg ggctcagacg ggtataggga 11280  
ccaggaagtc tcacaatccg atcattctga tatttcaggg catatttaggt ttggggtgca 11340  
aaggaagtac ttgggactta ggcacatgag actttgtatt gaaaatcaat gattggggct 11400  
35 ggccgtggtg ctcacgcctg taatctcatc actttggag accgaagtgg gaggatggct 11460  
tgatctcaag agttggacac cagcctaggc aacatggcca gaccctctct ctacaaaaaa 11520  
40 attaaaaatt agctggatgt ggtggtgcat gcttgggtc tcagctatcc tggaggctga 11580  
gacaggagaa tcgggtgagt ctgggagttc aaggctacag ggagctgcga tcacgcccgt 11640  
gcactccagc ctggaaaca gagtgagact gtctcagaat tttttaaaa aagaatcaat 11700  
45 gatcatccca accccctgttg ctgttcatcc tgagcctgccc ttctctggct ttgttcccta 11760  
gatcacatct ccatgatcca taggcctgc ccaatctgac ctcacaccgt gggaaatgcct 11820  
50 ccagactgat ctatgtatgtg tggaaacagca agtgcggct ctccctcccc ttccacagct 11880  
ctgggtgtgg gaggggggttg tccagcctcc agcagcatgg ggagggcctt ggtcagcata 11940  
taggtgccaa cagggcaagg gcggggctt ggagaatgaa ggctttagtgg ggtcctcag 12000  
55 ggaggcccccc cagccccaaa ctgcaccacc tggccgtgga caccgg 12047

## HRE-TRE SEQ ID NO:12

ccccgagg cagtgcattt gaggctcagg gcgtgcgtt gagtgcagcgagaccccg gggtgcag gccgga

5

## PSA-TRE SEQ ID NO:13

aagcttcttag ttttcttttc ccggtgacat cgtggaaagc actagcatct ctaagcaatg 60

10 atctgtgaca atattcacag ttaatgcca tccaggaaac tcaactgagc cttatgttcc  
120

agagatttttt gtgtttttt ctgagactga gtctcgctct gtgccaggct ggagtgcagt 180

15 ggtgcaaccc tggctcaactg caagctccgc ctctgggtt cacgccattc tcctgcctca 240

gcctcctgag tagctgggac tacaggcacc cggccaccacg cctggctaat tttttgtat 300

20 ttttagtaga gatggggttt cactgtgtta gccaggatgg tctcagtctc ctgacccctgt 360

gatctgccc ccttggcctc ccaaagtgc gggatgacag gcgtgagcca ccgcgcctgg 420

ccgatatacca gagatttttt gggggctcc atcacacaga catgttact gtcttcatgg 480

25 ttgactttta gtatccagcc cctctagaaa tctagctgat atagtgtggc tcaaaacctt 540

cagcacaat cacaccgtta gactatctgg tgtggccaa accttcaggta gaaaaagg 600

actctaattt ggcaggatac tccaaagcat tagagatgac ctcttgcaaa gaaaaagaaa 660

30 tggaaaagaa aaagaaaagaa aggaaaaaaaaaaaaaaa gagatgaccc ttcaggctct 720

gaggggaaac gcctgaggc tttgagcaag gtcagtcctc tttgcacag tctccctcac 780

35 agggtcattt tgacgatcaa atgtggtcac gtgtatgagg caccagcaca tgcctggctc 840

tggggagtgc cgtgttaagtg tatgcttgc ctgctgaatg gctggatgt gtcaggatt 900

40 atcttcagca cttacagatg ctcatctcat cctcacagca tcactatggg atgggtattta 960

ctggcctcat ttgtatggaga aagtggctgt ggctcagaaa gggggacca ctagaccagg 1020

gacactctgg atgctgggaa ctccagagac catgaccact caccaactgc agagaaattta 1080

45 attgtggcct gatgtccctg tcctggagag ggtggaggtg gacccctact aacccctac 1140

cttgaccctc tcttttaggg ctcttctga cctccaccat ggtacttagga cccattgtta 1200

ttctgtaccc tcttgactct atgacccca ccgcccactg catccagctg ggtccctcc 1260

50 tatctctatt cccagctggc cagtgcagtc tcagtgcacca cctgtttgtc agtaactctg 1320

aaggggctga cattttactg acttgcaaac aaataagcta actttccaga gttttgtaa 1380

55 tgctggcaga gtccatgaga ctcctgagtc agaggcaaag gcttttactg ctcacagctt 1440

agcagacagc atgaggttca tggtcacatt agtacacacccaaatctttag 1500

ggtgaccaga gcagtcatttggatgctgt gcagaagggg tttgtccac tggtagaaaa 1560  
cctgagatta ggaatcctca atcttatact gggacaactt gcaaacctgc tcagccttg 1620  
5 tctctgatga agatattatc ttcatgatct tggattgaaa acagacctac tctggaggaa 1680  
catattgtat cgattgtcct tgacagtaaa caaatctgtt gtaagagaca ttatctttat 1740  
tatctaggac agtaagcaag cctggatctg agagagatat catcttgc aaatgcctgc 1800  
10 tttacaaaca tccttgaaac aacaatccag aaaaaaaaaaag gtgttactgt ctttgctcag 1860  
aagacacaca gatacgtgac agaaccatgg agaattgcct cccaaacgctg ttcagccaga 1920  
15 gccttccacc ctttctgcag gacagtctca acgttccacc attaaatact tcttctatca 1980  
catcccgctt ctttatgcct aaccaaggtt cttagtcccg atcgactgtg tctggcagca 2040  
ctccactgcc aaaccaggaa taaggcagcg ctcaggatcc cgaaggggca tggctgggaa 2100  
20 tcagaacttc tgggtttgag tgaggagtgg gtccaccctc ttgaatttca aaggaggaag 2160  
aggctggatg tgaaggtaact gggggaggga aagtgtcagt tccgaactct taggtcaatg 2220  
25 agggaggaga ctggtaaggt cccagctccc gaggtactga tggggaaatg gcctaagaat 2280  
ctcatatcct caggaagaag gtgctggaat cctgagggggt agagttctgg gtatatttgc 2340  
ggcttaaggc tctttggccc ctgaaggcag aggctggaaac cattaggtcc agggtttggg 2400  
30 gtgatagtaa tgggatctct tgattcctca agagtctgag gatcgagggt tgccattct 2460  
tccatcttgc cacctaattcc ttactccact tgagggtatc accagccctt ctagctccat 2520  
35 gaaggcccc tgggcaagca caatctgagc atgaaagatg ccccagaggc cttgggtgtc 2580  
atccactcat catccagcat cacactctga ggggtggcc agcaccatga cgtcatgtt 2640  
40 ctgtgactat ccctgcagcg tgcctctcca gccacctgcc aaccgttagag ctgcccattcc 2700  
tcctctggtg ggagtggcct gcatggtgcc aggtgaggc ctgtgtcag acagggagcc 2760  
tgaatcata gggatccagg actcaaaagt gctagagaat ggccatatgt caccatccat 2820  
45 gaaatctcaa gggcttctgg gtggaggggca cagggacctg aacttatggt ttcccaagtc 2880  
tattgctctc ccaagtgagt ctcccagata cgaggcactg tgccagcatc agccttatct 2940  
ccaccacatc ttgtaaaagg actacccagg gccctgatga acaccatggt gtgtacagga 3000  
50 gtagggggtg gaggcacgga ctcctgtgag gtcacagcca agggagcatc atcatgggt 3060  
gggaggaggc aatggacagg cttgagaacg gggatgtgg tttctttgg 3120  
55 tagataaaagt gctgggtata ggattgagag tggagtatga agaccagtta ggatggagga 3180  
tcagattgga gttgggttag ataaagtgtt gggatagga ttgagagtgg agtatgaaga 3240  
ccagtttagga tggaggatca gattggagtt gggtagaga tgggtaaaa ttgtgctccg 3300

5            tagatttgtt ttgatgttgg ctcagacatc cttggggatt gaactgggaa tgaagctggg 3420  
tttgcattttg gaggtagaag acgtggaagt agctgtcaga tttgacagtg gccatgagtt 3480  
ttgtttgatg ggaaatcaaa caatggggaa agacataagg gttggcttgt tagtttaagt 3540  
10          tgcgttgggt tcatgggtc ggggctgtgt ataatgcagt tggattggtt tgtattaaat 3600  
tgggttgggt caggtttgg ttgaggatga gttgaggata tgcttggggaa caccggatcc 3660  
atgaggttct cactggagtg gagacaaact tccttccag gatgaatcca ggaaagcctt 3720  
15          aattcacgtg taggggaggt caggccactg gctaagtata tccttccact ccagctctaa 3780  
gatggtctta aattgtgatt atctatatcc acttctgtct ccctcactgt gcttggagtt 3840  
20          tacctgatca ctcaactaga aacagggaa gattttatca aattctttt tttttttttt 3900  
ttttttttag acagagtctc actctgttgc ccaggctgga gtgcagtggc gcagtctcgg 3960  
25          ctcactgcaa cctctgcctc ccaggttcaa gtgattctcc tgccctcagcc tcctgagttg 4020  
ctgggattac aggcatgcag caccatgccc agctaatttt tgtattttta gtagagatgg 4080  
gttttacca atgtttgcca ggctggcctc gaactcctga cctggtgatc cacctgcctc 4140  
30          agcctcccaa agtgcgtggaa ttacaggcgt cagccaccgc gcccagccac ttttgtcaaa 4200  
ttcttgagac acagctcggg ctggatcaag tgagctactc tggttttattt gaacagctga 4260  
aataaccaac ttttggaaa ttgatgaaat cttacggagt taacagtggaa ggtaccaggg 4320  
35          ctcttaagag ttcccgattt tcttctgaga ctacaaattt tgattttgca tgccacccca 4380  
atcttttttt tttttttttt aaatcgaggt ttcagttctca ttcttatttcc caggctggag 4440  
40          ttcaatagcg tgatcacagc tcactgttagc cttgaactcc tggccttaag agattctcct 4500  
gcttcggtct cccaatagct aagactacag tagtccacca ccatatccag ataattttta 4560  
aattttttgg gggccgggc acagtggctc acgcctgtaa tcccaacacc atgggaggct 4620  
45          gagatgggtg gatcacgagg tcaggagttt gagaccagcc tgaccaacat ggtgaaactc 4680  
tgtctctact aaaaaaaaaaaa aaaaatagaaaa aattagccgg gcgtggtggc acacggcacc 4740  
50          tgtaatccca gctactgagg aggctgaggc aggagaatca cttgaacccca gaaggcagag 4800  
gttgcaatga gccgagattt cggccactgca ctccagcctg ggtgacagag tgagactctg 4860  
tctcaaaaaaaaaaaa aaaaatttttt tttttttttt ttgttagagat ggatcttgct ttgtttctct 4920  
55          ggttggcctt gaactcctgg cttcaagtga tcctcctacc ttggcctgg aaagtgttgg 4980  
gattacaggc gtgagccacc atgactgacc tgcgttaat cttgaggtac ataaacctgg 5040

ctcctaaagg ctaaaggcta aatatttgtt ggagaagggg cattggattt tgcacatgagga 5100  
tgattctgac ctgggagggc aggtcagcag gcatctctgt tgcacagata gagtgtacag 5160  
5 gtctggagaa caaggagtgg ggggttattt gaattccaca ttgtttgctg cacgttggat 5220  
tttgaatgc taggaaactt tggagactc atatttctgg gctagaggat ctgtggacca 5280  
caagatctt ttatgtatgac agtagcaatg tatctgtgg gctggattct gggttgggag 5340  
10 tgcaaggaaa agaatgtact aaatgccaag acatctatcc caggagcatg aggaataaaaa 5400  
gttctagttt ctggtctcag agtggtgcat ggatcaggga gtctcacaat ctccctgagtg 5460  
15 ctgggtctt agggcacact gggtcttggg gtgcaaagga tctaggcacg tgaggcttg 5520  
tatgaagaat cggggatcgt acccaccctt tgtttctgtt tcatectggg catgtctcct 5580  
ctgcctttgt cccctagatg aagtctccat gagctacaag ggcctggtgc atccagggtg 5640  
20 atctagtaat tgcagaacag caagtgttag ctctccctcc ccttccacag ctctgggtgt 5700  
gggagggggt tgcaggcct ccagcagcat ggggagggcc ttggtcagcc tctgggtgcc 5760  
25 agcagggcag gggcggagtc ctggggaaatg aaggtttat agggctcctg ggggaggctc 5820  
cccagcccca agctt 5835

## CEA TRE SEQ ID NO:14

aagctttta	gtgctttaga	cagttagctg	gtctgtctaa	cccaagtgac	ctgggctcca	60
tactcagccc	cagaagtgaa	gggtgaagct	gggtggagcc	aaaccaggca	agcctaccct	120
cagggtccc	agtggcctga	gaaccattgg	accctaggacc	cattacttct	aggtaagga	180
aggtaacaaac	accagatcca	accatggtct	ggggggacag	ctgtcaaatg	cctazaaata	240
tacctggag	aggagcaggc	aaactatcac	tgccccaggt	tctctgaaca	gaaacagagg	300
ggcaacccaa	agtccaaatc	caggtgagca	ggtgcaccaa	atgcccagag	atatgacgag	360
gcaagaagtg	aaggaaaccac	ccctgcatca	aatgtttgc	atggaaagga	gaagggggtt	420
gctcatgttc	ccaatccagg	agaatgcatt	tggatctgc	cttcttcata	ctccctgggtt	480
agcaagacta	agcaaccagg	actctggatt	tggggaaaga	cgtttatttg	tggaggccag	540
tgatgacaat	ccacgaggg	cctaggtgaa	gagggcagga	aggctcgaga	cactggggac	600
tgagtgaaaa	ccacacccat	gatctgcacc	accatggat	gctccttcata	tgctcacctt	660
tctgttata	tcatgtggcc	ccatttctg	taccttcaca	gaaggacaca	ggctagggtc	720
tgtgcattggc	cttcatcccc	ggggccatgt	gaggacagca	ggggggaaag	atcatgggtc	780
ctcctgggtc	ctgcagggcc	agaacattca	tcacccatac	tgacctcata	gatggaaatg	840
gcttcctgg	ggctgggcca	acggggcctg	ggcaggggag	aaaggacgtc	aggggacagg	900
gaggaagggt	catcgagacc	cagcctggaa	ggttctgtc	tctgaccatc	caggattac	960
ttccctgcata	ctacctttgg	tcatttccc	tcaagcaatga	ccagctctgc	tccctgatct	1020
cagcctccca	ccctggacac	agcacccag	tccctggccc	ggctgcatacc	acccaaatacc	1080
ctgataaccc	aggaccatt	acttctaggg	taaggagggt	ccaggagaca	gaagctgagg	1140
aaaggctctga	agaagtca	tctgtcctgg	ccagggggga	aaaacccatca	gatgctgaac	1200
caggagaatg	ttgaccagg	aaaggacccg	aggacccaag	aaaggagtca	gaccacccagg	1260
gtttgcctga	gaggaaggat	caaggccccg	aggaaagca	gggctggctg	catgtgcagg	1320
acactggtgg	ggcatatgtg	tcttagattc	tccctgaatt	cagtgtccct	gccatggcca	1380
gactctctac	tcaggcctgg	acatgctgaa	ataggacaat	ggccttgc	tctctccca	1440
ccatggca	agagacataa	aggacattcc	aggacatgcc	ttccctggag	gtccagggttc	1500
tctgtctcac	acctcaggga	ctgtagttac	tgcattcagcc	atggtaggtg	ctgatctcac	1560
ccagcctgtc	caggcccttc	caactctccac	tttgtgacca	tgtccaggac	caccctcag	1620
atcctgagcc	tgcaaatacc	cccttgcgg	gtgggtggat	tcaagtaaaca	gtgggtcct	1680

atccagcccc gagagccacc tctgtcacct tctgtctggg catcatccca ctttcacaaag 1740  
 cactaaagag catggggaga cctggctagc tgggttctg catcacaaag aaaataatcc 1800  
 cccaggttcg gattcccagg gctctgtatg tggagctgac agacctgagg ccaggagata 1860  
 gcacaggtca gcccctaggga gggtggtca tccacccagg ggacagggtt gcaccagcc 1920  
 tgctactgaa agggcctccc caggacagcg ccatcagccc tgcctgagag ctttgctaaa 1980  
 cagcagtcag aggaggccat ggcagtggct gagctcctgc tccagggccc aacagaccag 2040  
 accaacagca caatgcagtc ctccccaaac gtcacaggc accaaaggaa aactgaggtg 2100  
 ctacctaacc ttagagccat caggggagat aacagccaa tttcccaaac aggccagttt 2160  
 caatccccatg acaatgaccc ctctgtctc attctccca aaataggacg ctgattctcc 2220  
 cccaccatgg atttctccct tgcctggga gcctttctg cccctatga tctggcact 2280  
 cctgacacac acctccctctc tggtgacata tcagggtccc tcactgtcaa gcagtccaga 2340  
 aaggacagaa ctttggacag cgcccatctc agcttcaccc ttccctccctc acagggttca 2400  
 gggcaaagaa taaatggcag aggccagtga gcccagagat ggtgacggc agtgcacccag 2460  
 gggcagatge ctggagcagg agctggcggg gccacaggga gaagggtatg caggaaggaa 2520  
 aacccagaaa tgggcagggaa aggaggacac aggctctgtg gggctgcagc ccagggttgg 2580  
 actatgatgt tgaagccatc tcagcaagta aggcaggc ccattaa gatggggagc 2640  
 acgtggcttc ctgtctgtatggggatggggatccat gccccataga accagatggc 2700  
 cggggttcag atggagaagg agcaggacag gggatccccca ggataggagg acocccagtgt 2760  
 ccccacccag gcaggtgact gatgaatggg catgcagggt cttccctggc tgggtctcc 2820  
 ctttgtccct caggattccct tgaaggaaca tccggaaagcc gaccacatct acctgggtggg 2880  
 ttctggggag tccatgtaaa gccaggagct tgggtgtctaa ggagggtca tggcatgtgc 2940  
 tggggcacc aaagagagaa acctgagggc aggcaggacc tggtctgagg aggcacatggg 3000  
 gcccagatgg ggagatggat gtcaggaaag gctgccccat cagggagggt gatagcaatg 3060  
 ggggtctgt gggagtggc acgtgggatt ccctggctc tgccaaagttc cctccatag 3120  
 tcacaacctg gggacactgc ccatgaaggg ggcctttgc ccagccagat gctgctgggt 3180  
 ctgccccatcc actaccctct ctgtccctc cactctgggt ctttctccag atgcctggaa 3240  
 cagccctggc ctgggcctgt cccctggagat gttgtgggag aagctgagtc tctggggaca 3300  
 ctctcatcag agtctgaaag gcacatcagg aaacatccct ggtctccagg actaggcaat 3360

gaggaaaggg ccccagctcc tccctttccc actgagaggg tcgaccctgg	3420
tgacttctgc gtctgtccca gtcacccctga aaccacaaca aaaccccccagc	3480
ccaggtacaa tacatgtggg gacagtctgt acccagggga agccagttct ctcttcctag	3540
gagaccgggc ctcaggcgt tgcccggggc aggcgggggc agcacgtgcc tgccttgag	3600
aactcgggac cttaagggtc tctgtctgt gaggcacagc aaggatcctt ctgtccagag	3660
atgaaagcag ctccctgcccc tcctctgacc tcttcctctt tcccaaatact caaccaacaa	3720
ataggtgttt caaatctcat catcaaatact tcatccatcc acatgagaaaa gctaaaaacc	3780
caatggattt acaacatcaa gagttggac aagtggacat ggagatgtta cttgtggaaa	3840
tttagatgtg ttcaagctatc gggcaggaga atctgtgtca aattccagca tggttcagaa	3900
gaatcaaaaaa gtgtcacagt ccaaataatgtgc aacagtgcag gggataaaaac tgggtgtcat	3960
tcaaaactgag ggatattttg gaacatgaga aaggaaggga ttgctgtgc acagaacatg	4020
gatgatctca cacatagagt taaaagaaag gagtcaatcg cagaatagaa aatgatcact	4080
aattccacat ctataaaat tccaagaggg aaacccaaatt ctgctgtctg agatcagaat	4140
ggaggtgacc tgccttgc aatggctgtg agggtcacgg gagtgtcaact tagtgcaggc	4200
aatgtgcgt atcttaatct gggcaggct ttcatgagca cataggaatg cagacattac	4260
tgctgtgttc attttacttc accggaaaaa aagaataaaa tcaagccgggc ggggtggctc	4320
acgcctgtaa tcccagact tttagaaggct gaggtgggca gattacttga ggtcaggagt	4380
tcaagaccac cctggccaaat atggtaaaaa cccggctcta ctaaaaatac aaaaattagc	4440
tgggcattgtt ggtgcgcgc tgtaatcccc gctactcggg aggctgaggg tggacaattt	4500
cttggaccca ggaagcagag gttgcagtga gccaaaggattg tgccactgca ctccagctt	4560
ggcaacagag ccagactctg taaaaaaaaaaa aaaaaaaaaaaa aaaaaaaaaaaa agazagaaaa	4620
agaaaaagaaa gtataaaaatc tctttgggtt aaaaaaaaaaaa gatccacaaa acaaacacca	4680
gctcttatca aacttacaca actctgccag agaacaggaa acacaaatac tcattaactc	4740
actttgtgg caataaaaacc ttcatgtca aaggagacca ggacacaatg aggaagtaaa	4800
actgcaggcc ctacttgggt gcagagaggg aaaatccaca aataaaaacat taccagaagg	4860
agctaagatt tactgcattg agttcatcc ccaggtatgc aagggtattt taacacactga	4920
aaatcaatca ttgcctttac tacatagaca gattagctag aaaaaaaaaaaa caactagcag	4980
aacagaagca atttggcctt cctaaaaatcc cacatcatat catcatgatg gagacagtgc	5040
agacgccaat gacaataaaa agagggacat ccgtcacccg gtaaacatgt ccacacagct	5100

ccagcaagca eccgtcttcc cagtgaatca ctctaaccctc ccctttaatc agccccaggc 5160  
 aaggctgcct gcgatggcca cacaggctcc aaccctgtgg cctcaacctc cccgagaggg 5220  
 tctccttgg ccacccatg gggagagcat gaggacaggg cagagccctc tcatgcccac 5280  
 acatggcagg agctgacgccc agagccatgg gggctggaga gcagagctgc tggggtcaga 5340  
 gcttcctgag gacacccagg cctaaggaa ggcagctccc tggatggggg caaccaggt 5400  
 cgggctcca acctcagagc cccatggga ggagccagca ctctaggcct ttccttagggt 5460  
 gactctgagg ggaccctgac acgacaggat cgctgaatgc acccgagatg aggggccac 5520  
 cacgggaccc tgctctcggt gcagatcagg agagagtggg acaccatgcc aggccccat 5580  
 ggcatggctg cgactgaccc aggcactcc cctgcatgca tcagcctcgg taagtcacat 5640  
 gaccaagccc aggaccaatg tggaaaggaag gaaacagcat ccccttttagt gatggAACCC 5700  
 aaggtcagtg caaagagagg ccatgagcag ttaggaaggg tggtccaacc tacagcaca 5760  
 accatcatct atcataagta gaagccctgc tccatgaccc ctgcattaa ataaacgttt 5820  
 gttaaatgag tcaaattccc tcaccatgag zgtcaacctg tgtgttaggcc catcacacac 5880  
 acaaacacac acacacacac acacacacac acacacacac acaggaaag tgcaggatcc 5940  
 tggacgac caggcaggct tcacaggcag zgczaacagc gtgaatgacc catgcagtgc 6000  
 cctggggccc atcagctcag agacccttgc zggctgaga tggggctagg caaggagag 6060  
 acttagagag ggtggggcct ccaggaggg ggctgcaggg agctgggtac tgcctccag 6120  
 ggagggggct gcaggagct gggtaactgcc ctccaggggag gggctgcag ggagctgggt 6180  
 actgcctcc agggaggggg ctgcaggag ctgggtactg cccctccaggg zgggggctgc 6240  
 agggagctgg gtactgcct ccaggaggc zggagcactg ttcccaacag zggatgcacatc 6300  
 ttccctgcagc agctgcacag acacagggc cccatgact gccctggcc zgggtgtgga 6360  
 ttcccaattt cgtccccat tgggtggac ggagggttgcac cgtgacatcc aaggggcatc 6420  
 tgtgattcca aacttaaact actgtgccta caaaatagga aataacccta ctttttctac 6480  
 tatctcaaat tccctaagca caagctagca ccctttaat caggaagttc agtcactcct 6540  
 ggggtctcc catgccccca gtctgacttg caggtgcaca ggggtggctga catctgtcct 6600  
 tgctcctcct cttggctcaa ctggccccc tccctgggggt gactgtatggt caggacaagg 6660  
 gatcctagag ctggcccccatttgcacagg zggcaggac ttggcctcca ttctgaagac 6720  
 taggggtgtc aagagagctg ggcacccac zggctgcac zggatgcgc ggacagaggg 6780

tgacacaggg ctcaggcgtt cagacgggtc gggaggctca gctgagagtt cagggacaga 6840  
cctgaggagc ctcagtggga aaagaagcac tgaagtggga agttctggaa tgttctggac 6900  
aaggctgagt gctctaagga aatgctccca ccccgatgtc gcctgcagca ctggacggtc 6960  
tgtgtacctc cccgctgccc atcctctcac agccccccgc tctagggaca caactcctgc 7020  
cctaacatgc atctttcttg tctcattcca cacaaaaggg cctctgggtt ccctgttctg 7080  
cattgcaagg agtggaggc acgttcccac agaccaccca gcaacagggt cctatggagg 7140  
tgcggtcagg aggatcacac gtccccccat gcccaggga ctgactctgg gggtgatgga 7200  
ttggcctggc ggccactggc cccctctgtc cctgaggggc atctgcaccc tggaggctgc 7260  
cacatccctc ctgattttt cagctgaggc cccttcttgc aatcccaggc aggactcaac 7320  
ccccactggg aaaggccccag tgtggacggc tccacagcag cccagctaa gcccctggac 7380  
acagatcctg zgtgagagaa cctttaggaa cacaggtgca cggccatgtc cccagtgc 7440  
acacagagca ggggcacatcg gaccctgagt gtgtagctcc cgcgactgaa cccagccctt 7500  
ccccatgac gtgacccctg gggtggtcc aggtctccag tccatgccac caaaatctcc 7560  
agattgaggc tcctcccttg agtccctgat gcctgtccag gagctgcccc ctgagcaat 7620  
ctagatgtca gagggctggg attgtggcag taaaagcagc cacattgtc tcaggaagga 7680  
aagggaggac atgagctcca ggaagggcga tggcgtcctc tagtggcgc ctccctgttaa 7740  
tgagcaaaaa ggggcacagga gagttgagag atcaggcgtg gccttggact aaggctcaga 7800  
tggagaggac tgaggtgcaaa agagggggct gaagtagggg agtggcggg agagatggga 7860  
ggagcaggta aggggaagcc ccaggggggc cggggggagg tacagcagag ctctccactc 7920  
ctcagcattt acattttgggg tggcgtgtc agtgggggtt tgtaagttgt agggtgttca 7980  
gcaccatctg gggactctac ccactaaatg ccagcaggac tccctccca agctctaaca 8040  
accaacaatg tctccagact ttccaaatgt cccctggaga gcaaaattgc ttctggcaga 8100  
atcaactgatc tacgtcagtc tctaaaatgt zctcatcagc gaaatccttc accttctggg 8160  
agaagaatca caagtgtgag aggggtagaa actgcagact tcaaaatctt tccaaaagag 8220  
tttacttaa tcagcagttt gatgtccag gagaagatac atttagagtg ttttagagt 8280  
atgccacatg gctgcctgtc cctcacagca ggagcagagt gggtttcca agggcctgtc 8340  
accacaactg gaatgacact cactgggtt cattacaaag tggaatgtgg ggaattctgt 8400  
agactttggg aagggaaatg tatgacgtga gcccacagcc taaggcagtg gacagtccac 8460  
tttggggctc tcaccatcta ggagacatct cagccatgaa catagccaca tctgtcatta 8520

gaaaacatgt-tttattaaga ggaaaaatct aggctagaag tgctt:atgc tctttttct 8580  
 ctttatgttc aaattcatat accttttagat cattccttaa agaacaatct atccccctaa 8640  
 gtaaatgtta tcactgactg gatagtgttgcgtgtctact cccaaacccct gtgtggtgac 8700  
 agtgcctgc ttccccagcc ctggggccctc tctgatttct gagagctttg ggtgtctcctt 8760  
 cattaggagg aagagaggaa ggggtttttt aatattctca ccattcaccc atccacctct 8820  
 tagacactgg gaagaatcag ttgcccactc ttggatttga tcctcgaatt aatgacactct 8880  
 atttctgtcc cttgtccatt tcaacaatgt gacaggccta aaggggtgcct tctccatgtg 8940  
 atttttgagg agaaggttct caagataagt ttctcacac ctcttgaat tacctccacc 9000  
 tgtgtccccca tcaccattac cagcagcatt tggacccttt ttctgttagt cagatgcttt 9060  
 ccacctcttg agggtgtata ctgtatgctc tctacacagg aatatgcaga gggaaatagaa 9120  
 aaaggaaat cgcattacta ttcagagaga agaagacctt tatgtgaatg aatgagagtc 9180  
 taaaatccta agagagccca tataaaatta ttaccagtgc taaaactaca aaagttacac 9240  
 taacagtaaa ctagaataat aaaacatgca tcacagttgc tggtaagct aaatcagata 9300  
 ttttttctt agaaaaagca ttccatgtgt gttcagtga tgcacaggagt gcccctcagt 9360  
 caatatgctg cctgttaattt ttgttccctg gcagaatgta ttgtctttc tccctttaaa 9420  
 tcttaaatgc aaaactaaag gcagctctg ggccccctcc ccaaagtcag ctgcctgcaa 9480  
 ccagccccac gaagagcaga ggcctgagct tccctggtca aaataggggg ctagggagct 9540  
 taaccttgct cgataaaagct gtgttccctg aatgtcgctc ctgttccctg gggcaccagc 9600  
 ctggagggtg gtgagcctca ctggtggctc gatgttacc ttgtgcctc acaccagtgg 9660  
 tcactggAAC cttgaacact tggctgtgc ccggatctgc agatgtcaag aacttctgga 9720  
 agtcaaatta ctgcccactt ctccaggcga gatacctgtg aacatccaaa accatgccac 9780  
 agaaccctgc ctggggctca caacacatat ggactgtgag caccaagtc agccctgaat 9840  
 ctgtgaccac ctgccaagat gcccctaact gggatccacc aatcactgca catggcaggc 9900  
 agcgaggctt ggaggtgctt cgcacaaagg cagccccat ttgctggag tttcttggca 9960  
 cctggtagtg gtgaggagcc ttgggaccct caggattact ccccttaagc atagtggga 10020  
 cccttctgca tccccagcag gtgccccct cttcagagcc tctctctctg aggtttaccc 10080  
 agaaccctgc accaatgaga ccatgtgaa gcctcagaga gagaatgga gctttgacca 10140  
 ggagccgctc ttccctgagg gcccaggcag ggaaagcagg aggcaaccc accgatggaa 10200

acaccagtgt ctaagccct gatgagaaca ggtggtctc tcccatatgc ccataccagg 10260  
cctgtgaaca gaatcctcct tctgcagtga caatgtctga gaggacgaca tgttccag 10320  
cctaacgtgc agccatgccc atctacccac tgcctactgc aggacagcac caacccagga 10380  
gctggaaagc tgggagaaga catggaatac ccatggcttc tcacccctt ccagtcagg 10440  
gggcaccatt tatgcctagg acacccaccc gcccggccca ggctcttaag agtttaggtca 10500  
cctaggtgcc tctgggaggc cgaggcagga gaattgcttg aacccgggag gcagaggttg 10560  
cagttagccg agatcacacc actgcactcc agcctgggtg acagaatgag actctgtctc 10620  
aaaaaaaaaaag agaaagatag catcagtggc taccaagggc tagggcagg ggaaggtgga 10680  
gagttaatga ttaatagtat gaagtttcta tgtgagatga tgaaaatgtt ctggaaaaaa 10740  
aaatatagtg gtgaggatgt agaatattgt gaatataatt aacggcattt aattgtacac 10800  
ttaacatgat taatgtggca tattttatct tatgtatttg actacatcca agaaacactg 10860  
ggagaggaa agcccaccat gtaaaataca cccaccctaa tcagatagtc ctcattgtac 10920  
ccaggtacag gcccctcatg acctgcacag gaataactaa ggatttaagg acatgaggct 10980  
tcccagccaa ctgcagggtgc acacataaa tgtatctgca aacagactga gagtaaagct 11040  
ggggcacaac acctcagcac tgccaggaca cacacccttc tcgtggattc tgactttatc 11100  
tgacccggcc cactgtccag atcttgggtg gggattgggaa caagggaggt cataaagct 11160  
gtccccaggg cactctgtgt gaggcaca gaccccttcc ccccccacc gttaggctc 11220  
cacacataga tctgaccatt aggcatgtg aggaggactc tagcggggc tcagggatca 11280  
caccaga gaa tcaggtacag agaggaagac gggctcgag gagctgtgg atgacacaga 11340  
gcagggttcc tgcagtcac aggtccagct caccctggtg taggtcccc atccccctga 11400  
tccaggcatc cctgacacag cccctcccg gagcctcctc ccaggtgaca catcagggtc 11460  
cctcaactcaa gctgtccaga gagggcagca cttggacag cccccccccc acttcactct 11520  
tcctccctca cagggctcag ggctcaggc tcaagtctca gaacaaatgg cagaggccag 11580  
tgagccaga gatggtgaca gggcaatgat ccagggcag ctgcctgaaa cgggagcagg 11640  
tgaagccaca gatgggagaa gatgggtcag gaagaaaaat ccaggaatgg gcaggagagg 11700  
agaggaggac acaggctctg tggggctgca gcccaggatg ggactaagtg tgaagacatc 11760  
tcagcagggtg aggccaggc ccatgaacag agaaggcagct cccacccccc ctgatgcacg 11820  
gacacacaga gtgtgtggtg ctgtcccccc agagtrgggc tctctgttc tggccccag 11880  
ggagtgagaa gtgagggtga cttgtccctg ctccctctctg ctaccccaac attcaccc 11940

tcctcatgcc-cctctctc aaatatgatt tggatctatg tccccgccca aatctcatgt 12000  
 caaattgtaa accccaaatgt tggaggtggg gccttgttag aagtgattgg ataatgcggg 12060  
 tggattttct gctttgatgc tgtttctgtg atagagatct cacatgatct ggttgtttaa 12120  
 aagtgtgtag cacctctccc ctctctctct ctctctctta ctcatgctct gccatgtaa 12180  
 acgttccgtt ttccccctca ccgtccagaa tgattgtaa 12240  
 agcagaagcc actatgttc ctgtacaact gcagaatgtat gagcgaatta aaccttttt 12300  
 ctttataaaat tacccagttt caggtatttc tttatagcaa tgcgaggaca gactaataca 12360  
 atcttctact cccagatccc cgacacacgt tagccccaga catcaactgcc cctgggagca 12420  
 tgcacagcgc agcctcctgc cgacaaaagc aaagtcacaa aaggtgacaa aaatctgcat 12480  
 ttggggacat ctgattgtga aagagggagg acagtacact ttagccaca gagactgggg 12540  
 ctcaccgagc tgaaacctgg tagcacttttgcataacatg tgcacatgaccgtgtttcaatg 12600  
 tcttagagatc agtgttgagt aaaaacagcct ggtctggggc cgctgtgtc cccacttccc 12660  
 tcctgtccac cagagggcgg cagagttcct cccaccctgg agcctccccca ggggctgtg 12720  
 acctccctca gcccggccca cagcccagca gggtecaccc tcacccgggt cacctcggcc 12780  
 cacgtcctcc tgcacctccg agtcctcac acggactctg tcaagtcctc cctgcagcc 12840  
 atcgccgccc cacctgagggc ttgtcggccg cccaaatttggag gcctgtcgcc tgcacctctgc 12900  
 aggcagctcc tgcacctac accccctcttcccccgggt cagctgaaag gggtctccc 12960  
 agggcagctc cctgtgatct ccaggacagc tcaagtcctc acaggctccg acgcccccta 13020  
 tgctgtcacc tcacagccct gtcattacca ttaacttcctc agtcccattga agttcaactga 13080  
 gcgcctgtct cccgggttaca gaaaaactct gtgacaggga ccacgtctgt cctgtctct 13140  
 gtggaaatccc agggcccagc ccagtgcctg acacggaaaca gatgctccat aaatactgtt 13200  
 taaaatgtgtg ggagatctt aaaaagaagc atatcacctc cgtgtggccc ccagcagtca 13260  
 gagtctgttc catgtggaca cagggcact ggcaaccagca tgggaggagg ccagcaagt 13320  
 cccgcggctg ccccaaggaat gaggcctcaa ccccaagagc ttcagaaggg aggacagagg 13380  
 cctgcaggga atagatcctc cggcctgacc ctgcagccata atccagagtt cagggtca 13440  
 tcacaccacg tcgacctgg tcaagcatccc tagggcagtt ccagacaagg ccggaggct 13500  
 cctcttgcac tccaggggtt gacattgcac acagacatca ctcagaaac ggattccct 13560  
 ggacacggaaatcggctttgc taaggaagtggagc ctggttccca tcccttgc 13620

caacagaccc ttctgatctc tcccacatac ctgctctgtt cctttctggg tcctatgagg 13680  
 accctgttct gccaggggtc cctgtgcaac tccagactcc ctccctggcac caccatgggg 13740  
 aagggtgggt gatcacagga cagtcagccct cgcagagaca gagaccaccc aggactgtca 13800  
 gggagaacat ggacaggccc tgagccgcag ctcagccaac gagacacggag agggagggtc 13860  
 cccctggagc cttccccaag gacagcagag cccagagtca cccacacctcc tccaccacag 13920  
 tcctctcttt ccaggacaca caagacaccc ctccctccac atgcaggatc tgggactcc 13980  
 tgagacctct gggcctgggt ctccatccct ggtcagtgg cgggggttggt ggtactggag 14040  
 acagagggtct ggtccctccc cagccaccac ccagtgagcc tttttcttagc ccccaagagcc 14100  
 acctctgtca ctttctgtt gggcatcatc ccacccccc agagccctgg agagcatggg 14160  
 gagacccccc accctgctgg gtttctctgt cacaaaggaa aataatcccc ctgggtgtgac 14220  
 agacccaagg acagaacaca gcagaggtca gcactggga gagacaggttg tcctccagg 14280  
 ggtatgggggt ccatccaccc tgccgaaaag atttgtctga ggaactgaaa atagaaggaa 14340  
 aaaaagagga gggacaaaag aggacaaaat gagaggggag gggacagagg acacctgaat 14400  
 aaagaccaca cccatgaccc acgtgatgct gagaagtact cctgccctag gaagagactc 14460  
 transcription start site  
 agggcagagg gaggaggac agcagaccag acagtcacag cagccttgc aaaaacgttcc 14520  
 tggaactcaa gctttctcc acagaggagg acagagcaga cagcagagac catggagtct 14580  
 ccctcggccc ctccccacag atggtgcaccc ccctggcaga ggctcctgtct cacaggtgaa 14640  
 gggggacaa cctgggagag ggtgggagga gggagctggg gtctcctgg tagggcagg 14700  
 ctgtgagacg gacagaggc tcctgttggc gcctgaatacg ggaagaggac atcagagagg 14760  
 gacagggagtc acaccagaaa aatcaattt aactggatt gaaaggccc aggaaaacct 14820  
 caagagttct attttcttag ttaattgtca ctggccacta cttttttaaa aatcataata 14880  
 actccatcag atgacacttt aaataaaaac ataaccaggc catgaaacac tgcctcatc 14940  
 cgcctaccgc ggacatttggc aaataagccc caggctgtgg agggccctgg gaaccctcat 15000  
 gaactcatcc acaggaatct gcagcctgtc ccaggcactg ggtgcaacc aagatc 15056

**Mucin-TRE SEQ ID NO: 15**

5 cgagcggccc ctcagcttcg gcgcccagcc ccgcaaggct cccggtgacc actagagggc 60  
gggaggagct cctggccagt ggtggagagt ggcaaggaag gacccttaggg ttcatcgag 120  
cccaggtta ctcccttaag tggaaatttc ttccccact ctccttggc tttctccaag 180  
10 gagggAACCC aggctgctgg aaagtccggc tggggcgggg actgtgggtt caggggagaa 240  
cggggtgtgg aacgggacag ggagcggta gaagggtggg gctattccgg gaagtgggtgg 300  
ggggagggag cccaaaacta gcacctagtc cactcattat ccagccctct tatttctcg 360  
15 ccgctctgct tcagtggacc cggggagggc ggggaagtgg agtgggagac ctagggtgg 420  
gttcccgac cttgctgtac aggacctcga cctagctggc ttgttcccc atccccacgt 480  
20 tagttgttgc cctgaggcta aaactagagc ccagggccc caagttccag actgcccctc 540  
ccccctcccc cggagccagg gagtggttgg taaaaggggg aggccagctg gagaacaaac 600  
ggtagtcag ggggttgagc gattagagcc ctgttaccct acccaggaat gttggggag 660  
25 gaggaggaag aggtaggagg taggggaggg ggcggggttt tgtcacctgt cacctgctcg 720  
ctgtgcctag ggcgggcggg cggggagtg ggaccgggt ataaagcggt aggccctgt 780  
30 gcccgcctca cctctcaagc agccagcgcc tgcctgaatc tggttgc cctccccacc 840  
catttcacca ccaccatg 858

35

**αFP-TRE SEQ ID NO:16**

40 gaattcttag aaatatgggg gtaggggtgg tggtggtaat tctgtttca ccccataggt 60  
gagataagca ttgggttaaa tgtgcttca cacacacatc acatttcata agaattaagg 120  
45 aacagactat gggctggagg actttgagga tgtctgtctc ataacacttg gtttgtatct 180  
gttctatggg gcttgttta agcttggcaa cttgcaacag gttcaactga ctttctcccc 240  
aagcccaagg tactgtcctc ttttcatatc tggggggg cctctggggc ttgaatatct 300  
50 gagaaaaat aaacatttca ataatgttct gtggtgagat gagtatgaga gatgtgtcat 360  
tcatttgtat caatgaatga atgaggacaa ttagtgtata aatccttagt acaacaatct 420  
55 gagggtaggg gtggtactat tcaatttcta tttataaaga tacttatttc tatttattta 480  
tgcttgtgac aaatgttttgc ttggggacca caggaatcac aaagatgagt ctttqaattt 540

aagaagttaa tggtccagga ataattacat agcttacaaa tgactatgt ataccatcaa 600  
acaagaggtt ccatgagaaa ataatctgaa aggttaata agttgtcaaa ggtgagaggg 660  
5 ctcttctcta gctagagact aatcagaaat acattcaggg ataattattt gaatagacct 720  
taagggttgg gtacattttg ttcaagcatt gatggagaag gagagtgaat atttgaaaac 780  
10 atttcaact aaccaaccac ccaatccaac aaacaaaaaa tgaaaagaat ctcagaaaca 840  
gtgagataag agaaggaatt ttctcacaac ccacacgtat agctcaactg ctctgaagaa 900  
gtatatatct aatatttaac actaacatca tgctaataat gataataatt actgtcattt 960  
15 ttaatgtct ataagtacca ggcatttaga agatattatt ccatttat atcaaataaa 1020  
acttgagggg atagatcatt ttcatgatat atgagaaaaa ttaaaaacag attgaattat 1080  
20 ttgcctgtca tacagctaatt aattgaccat aagacaatta gatttaaatt agtttgaat 1140  
ctttctaata ccaaagttca gtttactgtt ccatgttgc tctgagtggc ttcacagact 1200  
tatgaaaaag taaacggaat cagaattaca tcaatgcaaa agcattgctg tgaactctgt 1260  
25 acttaggact aaactttgag caataacaca catagattga ggattgtttg ctgttagcat 1320  
acaaactctg gttcaaagct ccttttatt gttgtcttgc gaaaatttgc ttttcttcat 1380  
ggtttctctt ttcaactgcta tctattttc tcaaccactc acatggctac aataactgtc 1440  
30 tgcaagctta tgattccaa atatctatct ctgcctcaa tcttgccttca gaagataaaa 1500  
agtagtattc aaatgcacat caacgtctcc acttggaggg cttaaagacg tttcaacata 1560  
35 caaaccgggg agtttgcct ggaatgttc ctaaaatgtg tcctgttagca catagggtcc 1620  
tcttgcctt taaaatctaa ttacttttag cccagtgc tccacacta tggggagatg 1680  
40 agagtgaaaa gggagcctga ttaataatta cactaagtca ataggcatag agccaggact 1740  
gtttggtaa actggtcact ttatctaaa ctaaatataat ccaaactga acatgtactt 1800  
agttactaag tcttgactt tatctcattt ataccactca gctttatcca ggccacttat 1860  
45 ttgacagtat tattgcggaa acttcctaact tggtctcctt atcatagtct tatccccctt 1920  
tgaaacaaaa gagacagttt caaaatacaat atatgatttt tattagctcc cttttgttgt 1980  
50 ctataatagt cccagaagga gttataactt ccattttaaa agtcttgag atgtggccct 2040  
tgccaaactt gccaggaatt cccaatatct agtattttctt actattaaac tttgtgcctc 2100  
ttcaaaaactg cattttctctt cattccctaa gtgtgcattt tttccctta ccgggttggtt 2160  
55 tttccaccac ctttacatt ttcttggaaactataaccct cccttcat ttggcccacc 2220  
tctaattttc tttcagatct ccatgaagat gttacttcctt ccaggaagcc ttatctgacc 2280  
cctccaaaga tgtcatgagt ttcttttttctt attctactaa tcacagcatc catcacacca 2340

tgttgtgatt actgatacta ttgtctgttt ctctgattag gcagtaagct caacaagagc 2400  
5 tacatggtgc ctgtctcttg ttgctgatta ttcccatcca aaaacagtgc ctggaatgca 2460  
gacttaacat tttattgaat gaataaataa aaccccatct atcgagtgct actttgtgca 2520  
agacccgggtt ctgaggcatt tatatttatt gatttattta attctcattt aaccatgaag 2580  
10 gaggtactat cactatcctt attttatagt tgataaagat aaagcccaga gaaatgaatt 2640  
aactcaccca aagtcatgta gctaagtgac agggcaaaaa ttcaaaccag ttcccccaact 2700  
15 ttacgtgatt aatactgtgc tatactgcct ctctgatcat atggcatgga atgcagacat 2760  
ctgctccgta aggcagaata tggaggaga ttggaggatg acacaaaacc agcataatat 2820  
cagaggaaaa gtccaaacag gacctgaact gatagaaaaag ttgttactcc tggtagtc 2880  
20 gcatcgacat cttgatgaac tggcgttga cacaacatac attggcttga tggtagata 2940  
ttattttagt ttgtgtgtt attttatat atatattgt aatattgaaa tagtcataat 3000  
25 ttactaaagg cctaccattt gccaggcatt tttacatttgc tccctctaa tctttgatg 3060  
agatgatcag attggattac ttggccttga agatgatata tctacatcta tatctatatc 3120  
tatatctata tctatatcta tatctatatc tatatctata tatgtatatc agaaaagctg 3180  
30 aaatatgttt tgtaaagtta taaagatttc agactttata gaatctggga tttgccaaat 3240  
gtaacccctt tctctacatt aaacccatgt tggacaaat acatttatta ttcattcatac 3300  
35 aaatgttgct gagtcctggc tatgaaccag acactgtgaa agcctttggg atatttgcc 3360  
catgcttggg caagcttata tagtttgctt cataaaactc tatttcagtt cttcataact 3420  
aatacttcat gactattgct tttcaggtat tccttcataa caaatacttt ggctttcata 3480  
40 tatttgagta aagtccccct tgaggaagag tagaagaact gcactttgta aatactatcc 3540  
tggaatccaa acggatagac aaggatggtg ctacctttt ctggagagta cgtgagcaag 3600  
45 gcctgttttgc ttaacatgtt ccttaggaga caaaacttag gagagacacg catagcagaa 3660  
aatggacaaa aactaacaaa tgaatggaa ttgtacttga ttagcattga agaccttgg 3720  
tatactatga taaatgtttg tatttgctgg aagtgtact gacggtaaac ccttttgg 3780  
50 taaatgtgtg ccctagtagc ttgcagttatg atctatttt taagtactgt acttagctt 3840  
tttaaaaatt ttatgtttaa aattgcatac tgctttca ttgaagaagt tttgagagag 3900  
agatagaatt aaattcactt atcttaccat ctagagaaac ccaatgttaa aactttgtt 3960  
55 tccattatcc ctgtctttta ttcaacatcc ttttagagg gtgggaggaa tacagaggag 4020  
gtacaatgat acacaaatga gagcactctc catgtattgt tttgtccctgt tttcagtt 4080

acaatatatt atgagcatat ttccatttca ttaaatattc ttccacaaag ttatTTTgat 4140  
ggctgtatat caccctactt tatgaatgta ccatattaat ttatTCCTg gtgtgggtta 4200  
5 tttgatttta taatcttacc tttagaataa tgaaacacct gtgaagctt agaaaatact 4260  
ggtgccctggg tctcaactcc acagattctg atttaactgg tctgggttac agactaggca 4320  
ttgggaattc aaaaagttcc cccagtgatt ctaatgtgta gccaagatcg ggaacccttg 4380  
10 tagacaggga tgataggagg tgagccactc ttagcatcca tcatttagta ttaacatcat 4440  
catcttgagt tgctaagtga atgatgcacc tgaccactt tataaagaca catgtgcaaa 4500  
15 taaaattatt ataggacttg gtttatttagg gcttgTgctc taagtttct atgttaagcc 4560  
atacatcgca tactaaatac tttaaaatgt accttattga catacatatt aagtgaaaag 4620  
20 tgTTTCTgag ctaaacaatg acagcataat tatcaagcaa tgataatttgc aaatgaattt 4680  
attattctgc aacttaggga caagtcatct ctctgaattt ttgtacttt gagagtattt 4740  
gttatatttg caagatgaag agtctgaattt ggtcagacaa tgtcttggt gcctggcata 4800  
25 tgataggcat ttaatagttt taaagaatta atgtatttag atgaattgca taccaaattct 4860  
gctgtctttt ctTtatggct tcattaactt aattttagag aaattaatta ttctgcaact 4920  
tagggacaag tcatgtctttt gaatattctg tagTTTgagg agaatatttgc 4980  
30 aaaataaaat aagtttgcaa gttttttttt tctgccccaa agagctctgt gtccttgaac 5040  
ataaaaataca aataaccgct atgctgttaa ttattggcaa atgtcccatt ttcaacctaa 5100  
35 ggaaatacca taaagtaaca gatataccaa caaaaggtaa ctagttaaca ggcattgcct 5160  
gaaaagagta taaaagaattt tcagcatgat ttccatattt gtgcttccac cactgccaat 5220  
40 aaca 5224

CLAIMS

What is claimed is:

1. A replication-competent adenovirus vector comprising first and second genes co-transcribed as a single mRNA wherein the first and the second genes are under transcriptional control of a heterologous, target cell-specific transcriptional regulatory element (TRE), wherein the second gene has a mutation in or deletion of its endogenous promoter and is under translational control of an internal ribosome entry site (IRES) and wherein said vector exhibits greater specificity for the target cell than an adenovirus vector comprising a target cell-specific TRE operably linked to a gene and lacking an IRES.
2. The vector of Claim 1, wherein at least one of said first and second genes is an adenovirus gene.
- 15 3. The vector of Claim 2, wherein both of said first and said second genes are adenovirus genes.
4. The vector of Claim 2, wherein at least one of said first and said second adenovirus gene is essential for viral replication.
- 20 5. The vector of Claim 4, wherein the adenovirus gene essential for viral replication is an adenovirus early gene.
6. The vector of Claim 5, wherein the adenovirus early gene includes E1A, E1B, E2, or E4.
7. The vector of Claim 4, wherein the adenovirus gene essential for viral replication is an adenovirus late gene.
- 30 8. The vector of Claim 3, wherein both said first and said second adenovirus genes are essential for viral replication.

9. The vector of Claim 8, wherein at least one of said first and said second adenovirus genes is an adenovirus early gene.

10. The vector of Claim 8, wherein at least one of said first and said second 5 adenovirus genes is an adenovirus late gene.

11. The vector of Claim 8, wherein said first adenovirus gene is E1A and said second adenovirus gene is E1B.

10 12. The vector of Claim 11 wherein E1A has its endogenous promoter deleted.

13. The vector of Claim 11 wherein E1A has an inactivation of E1A enhancer I

15 14. The vector of Claim 11 wherein E1B has an inactivation of its endogenous promoter.

15. The vector of Claim 11 wherein E1B has a deletion of the 19-kDa region.

20 16. The vector of Claim 11 wherein E1A has an inactivation of its endogenous promoter and E1B has an inactivation of its endogenous promoter.

17. The vector of Claim 16 wherein E1B has a deletion of the 19-kDa region.

25 18. The vector of Claim 16 wherein E1A has an inactivation of E1A enhancer I.

19. The vector of Claim 1, wherein the internal ribosome entry site (IRES) is from EMCV.

30 20. The vector of Claim 1 wherein the IRES is from VEGF.

21. The vector of Claim 1 wherein the IRES includes the 5'UTR of HCV; the 5' UTR of BiP; or the 5'UTR of PDGF.

22. The vector of Claim 1, wherein the TRE is specific for a target cell that is a cancer cell.

5 23. The vector of Claim 22 wherein the cancer cell includes a prostate cancer cell, a breast cancer cell, a hepatoma cell, a melanoma cell, a bladder cell or a colon cancer cell.

10 24. The vector of Claim 22, wherein the TRE includes the probasin (PB) TRE, the prostate-specific antigen (PSA) TRE, the mucin (*MUC1*) TRE, the  $\alpha$ -fetoprotein (AFP) TRE, the *hKLK2* TRE, the tyrosinase TRE, the human uroplakin II (hUPII) TRE or the carcinoembryonic antigen (CEA) TRE.

15 25. The vector of Claim 9 wherein said first adenovirus gene has a deletion of its endogenous promoter.

26. The vector of Claim 25 wherein said first adenovirus gene is E1A.

20 27. The vector of Claim 9 wherein said first and/or said second adenovirus gene has a deletion of an enhancer region.

28. The vector of Claim 27 wherein said first gene is E1A and said enhancer is E1A enhancer I.

25 29. The vector of Claim 1 wherein said TRE has an endogenous silencer element deleted.

30 30. The vector of Claim 1 wherein said adenovirus vector comprises an E3 region.

30

31. The adenovirus vector of Claim 11 wherein said adenovirus comprises an E3 region.

32. The adenovirus vector of Claim 11 further comprising a transgene.
33. An adenovirus vector comprising a gene under transcriptional control of a melanocyte-specific TRE.  
5
34. The vector of Claim 33 wherein said gene is an adenoviral gene.
35. The vector of Claim 34 wherein said adenoviral gene is a gene essential for replication.  
10
36. The vector of Claim 32 wherein said transgene is co-transcribed with said first and said second gene and said transgene is under the translation control of a separate internal ribosome entry site (IRES).  
15
37. The vector of Claim 36 wherein said IRES is from EMCV.
38. The vector of Claim 36 wherein said IRES is from VEGF.
39. The vector of Claim 30 further comprising an adenovirus death protein gene  
20 (ADP).  
25
40. The vector of Claim 32 wherein said transgene is a cytotoxic gene.
41. The vector of Claim 1 wherein said first adenovirus gene is essential for viral replication and said second adenovirus gene is the adenovirus death protein gene (ADP).  
42. The vector of Claim 41 wherein said first adenovirus gene is E1A.  
30
43. The vector of Claim 42 wherein E1A has a deletion of its endogenous promoter.  
44. The vector of Claim 42 wherein said E1A has a deletion of E1A enhancer I.

45. The vector of Claim 1 wherein said first gene is essential for viral replication and said second gene is E3.

5 46. The vector of Claim 45 wherein said first gene is E1A.

47. A composition comprising a vector according to Claim 1.

10 48. The composition of Claim 47 further comprising a pharmaceutically acceptable excipient.

49. A composition comprising a vector according to Claim 30.

15 50. The composition of Claim 49 further comprising a pharmaceutically acceptable excipient.

51. A host cell comprising the vector of Claim 1.

52. A host cell comprising the vector of Claim 30.

20 53. An adenovirus vector comprising E1B under transcriptional control of a heterologous, target cell specific TRE, wherein E1B has a deletion of part or all of the 19-kDa region.

25 54. A host cell comprising the adenovirus vector of claim 53.

55. A method for propagating a replication-competent adenovirus vector comprising a target cell-specific TRE, said method comprising combining an adenovirus vector of Claim 1 with mammalian cells that permit the function of a target cell-specific TRE, such that the adenovirus vector enters the cell, whereby said adenovirus vector is propagated.

56. A method for conferring selective cytotoxicity in target cells, comprising contacting the cells with an adenovirus vector of Claim 41 whereby the vector enters the cell.

5 57. A method for modifying the genotype of a target cell, comprising contacting the cell with an adenovirus vector of Claim 1, wherein the vector enters the cell.

10 58. A method for suppressing tumor cell growth, comprising contacting a tumor cell with an adenovirus vector of Claim 41 such that the adenovirus vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell.

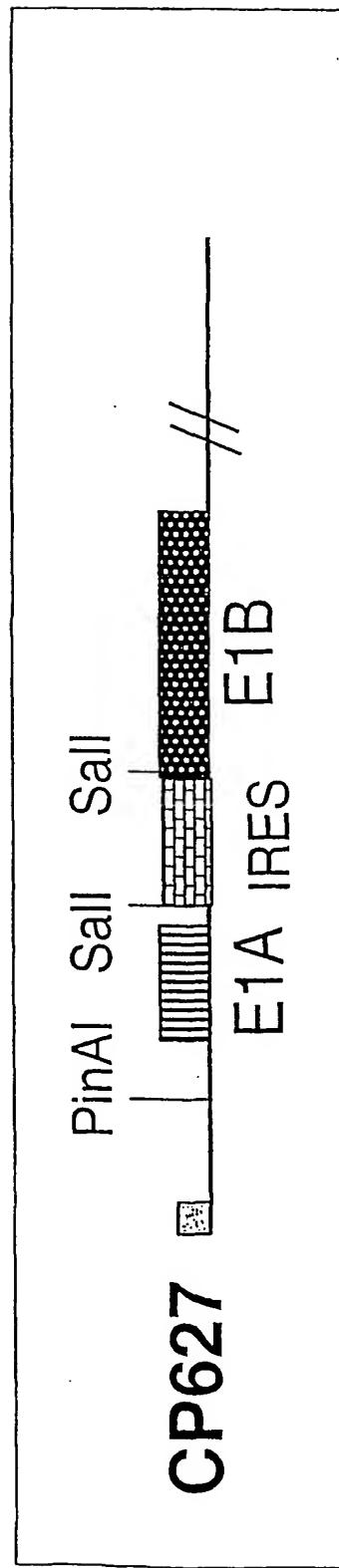
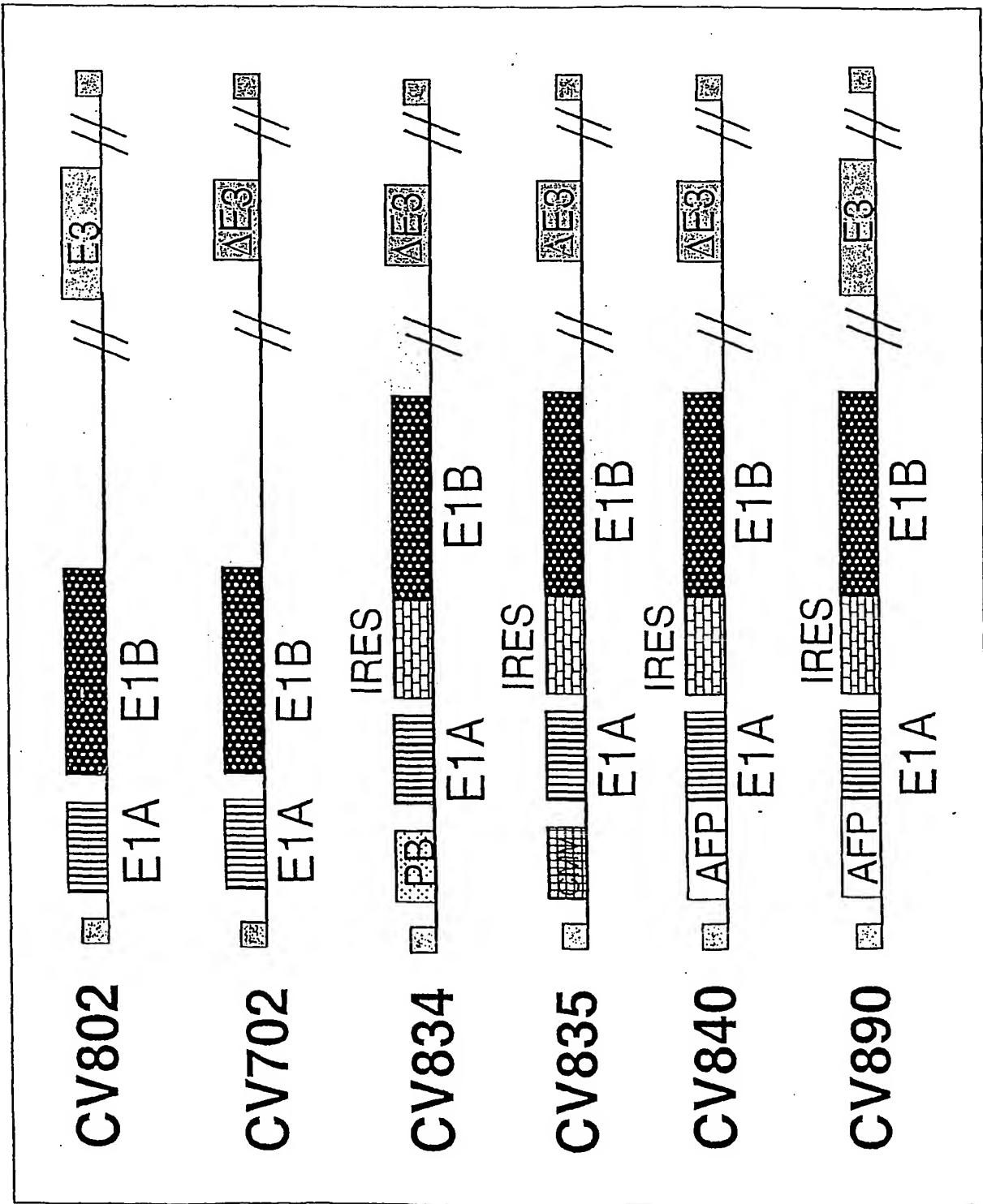


Figure 1

Figure 2A



# Structure of CV876, CV882 and CV884

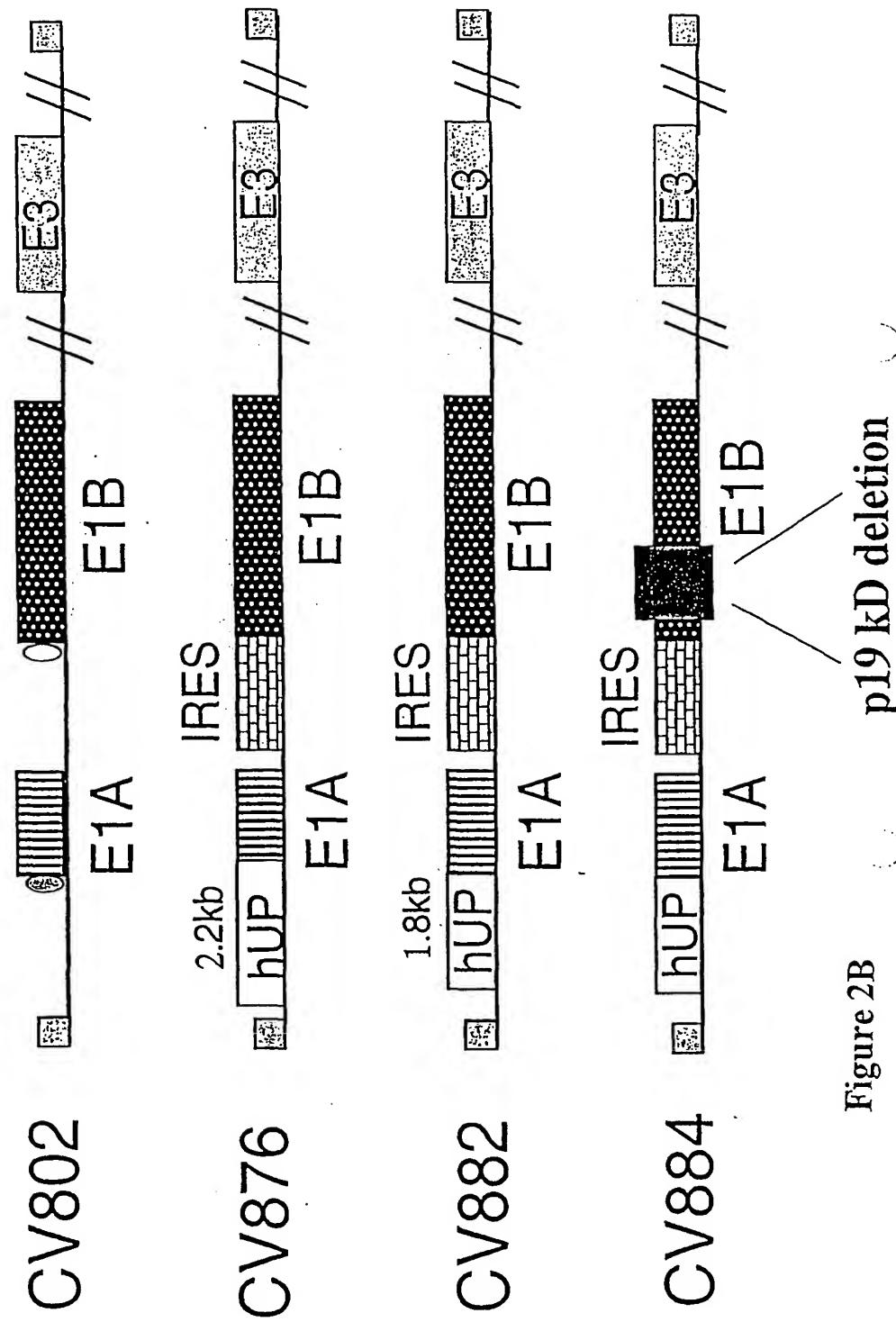


Figure 2B

## REPLICATION

293 LNCap HepG2

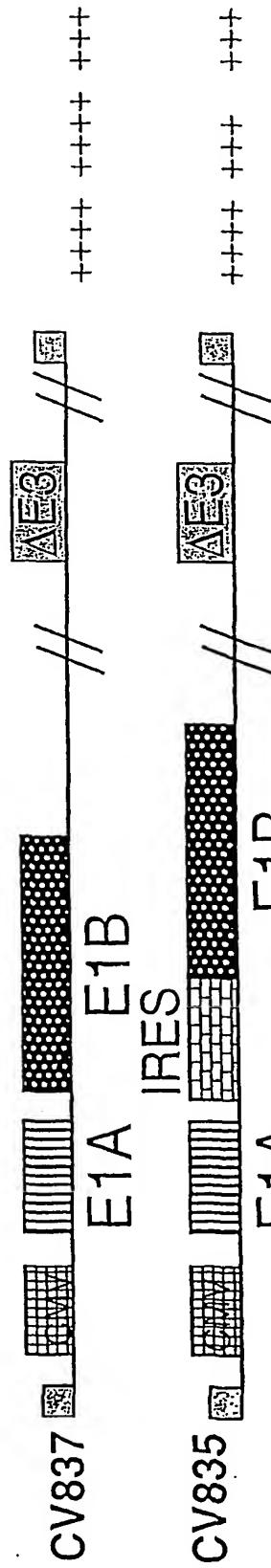
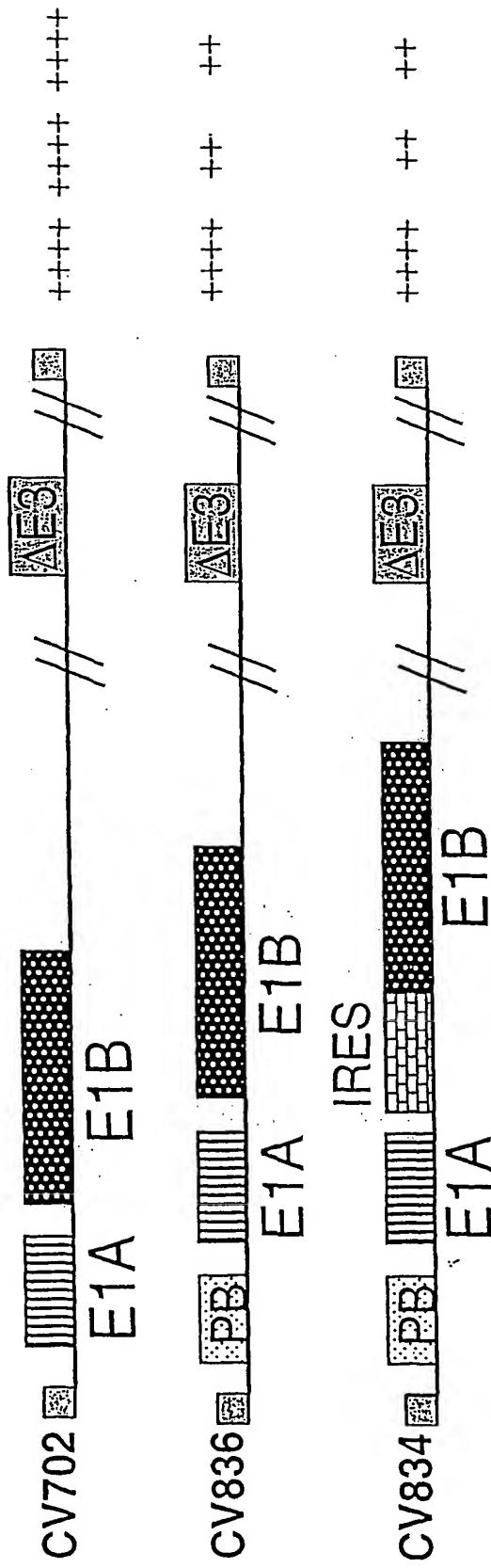


Figure 3

Figure 4A

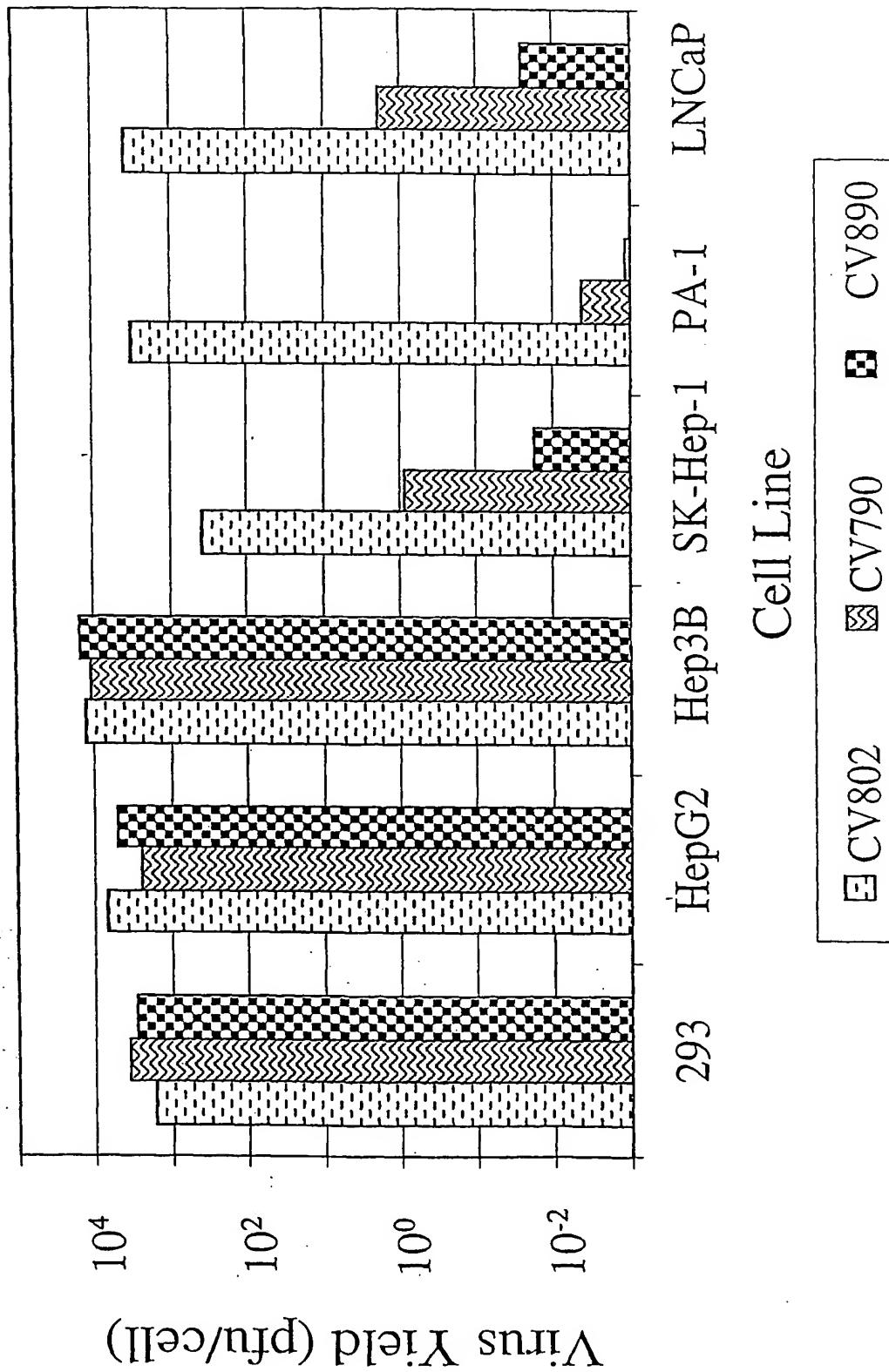
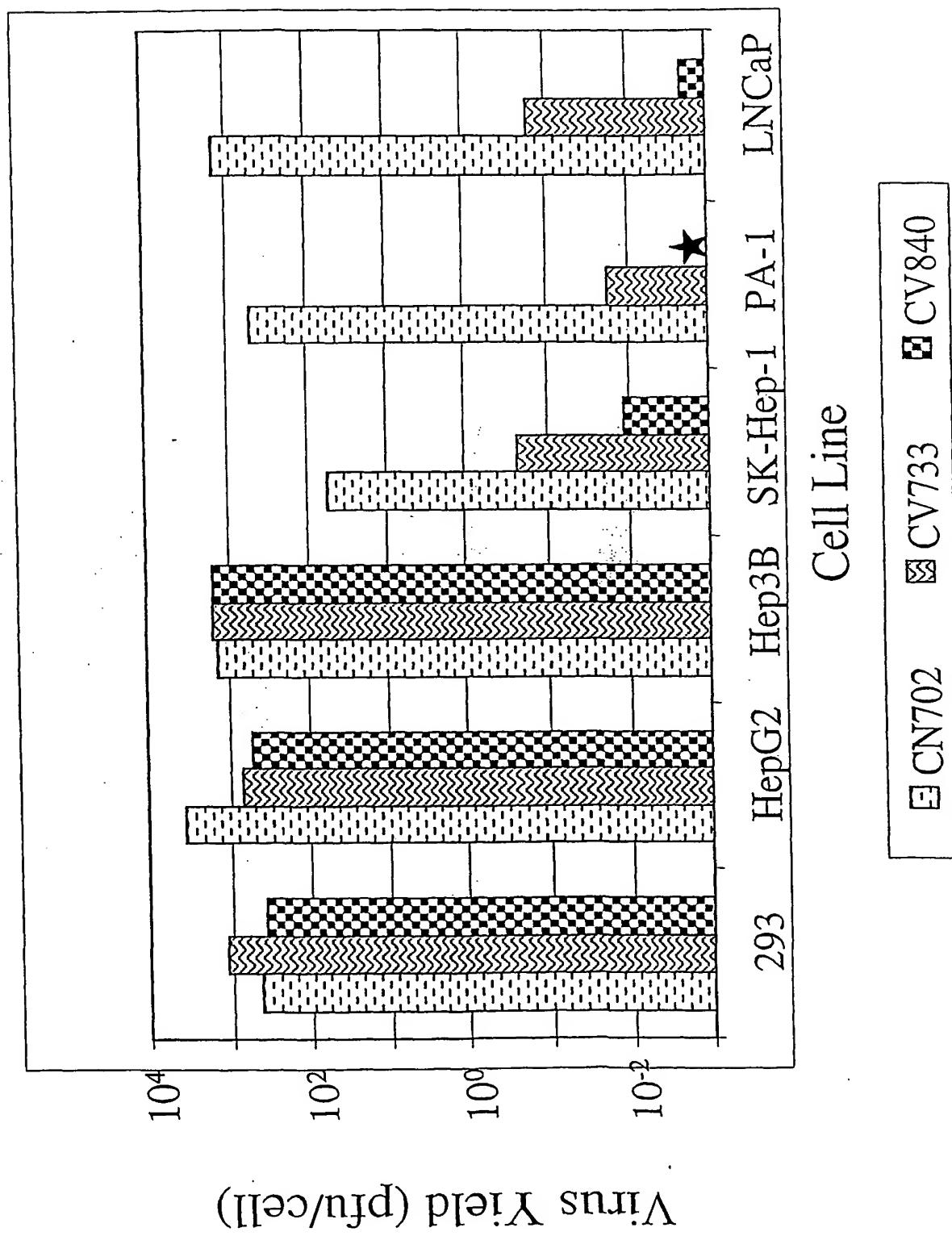


Figure 4B



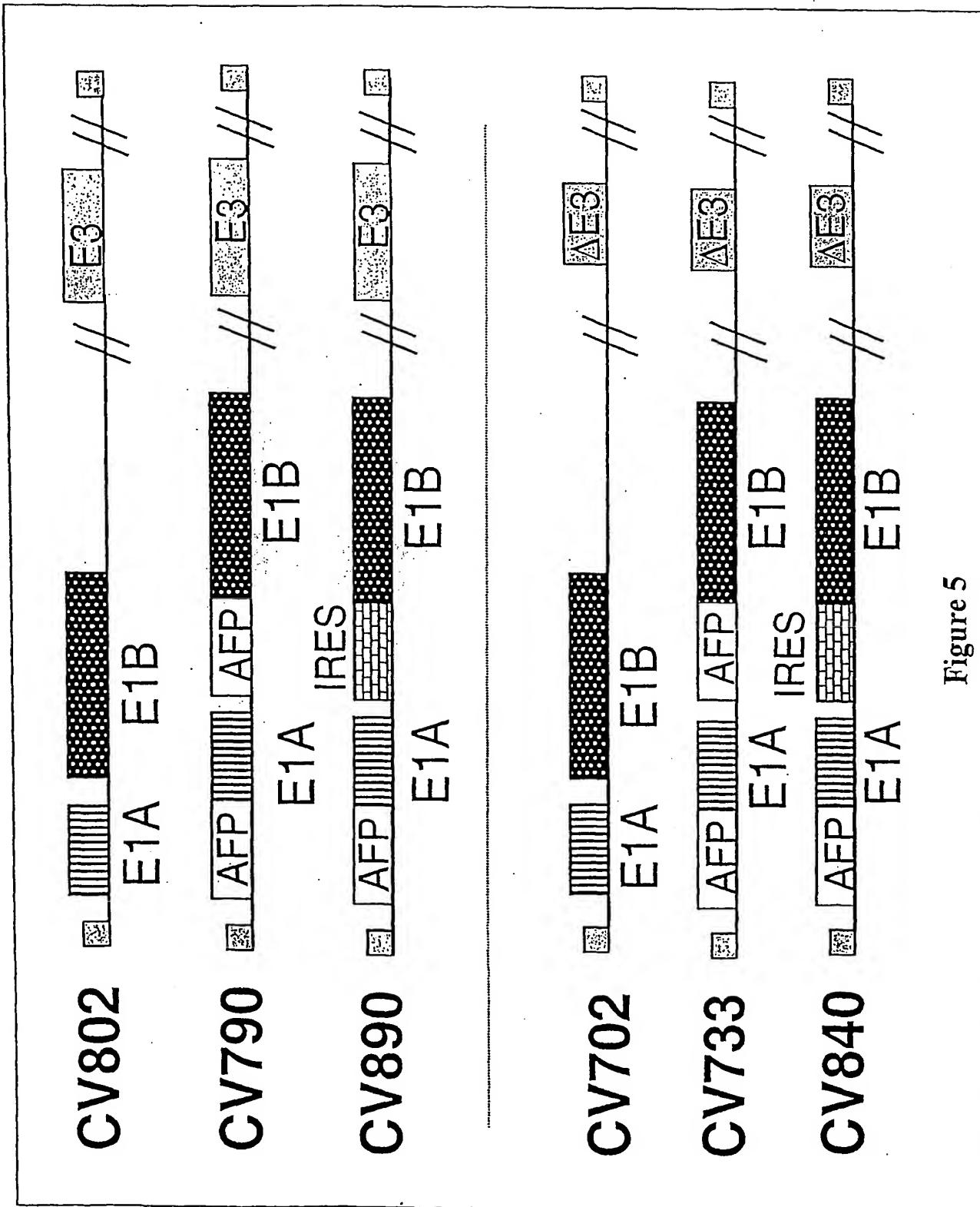


Figure 5

# Region E3 of Adenovirus

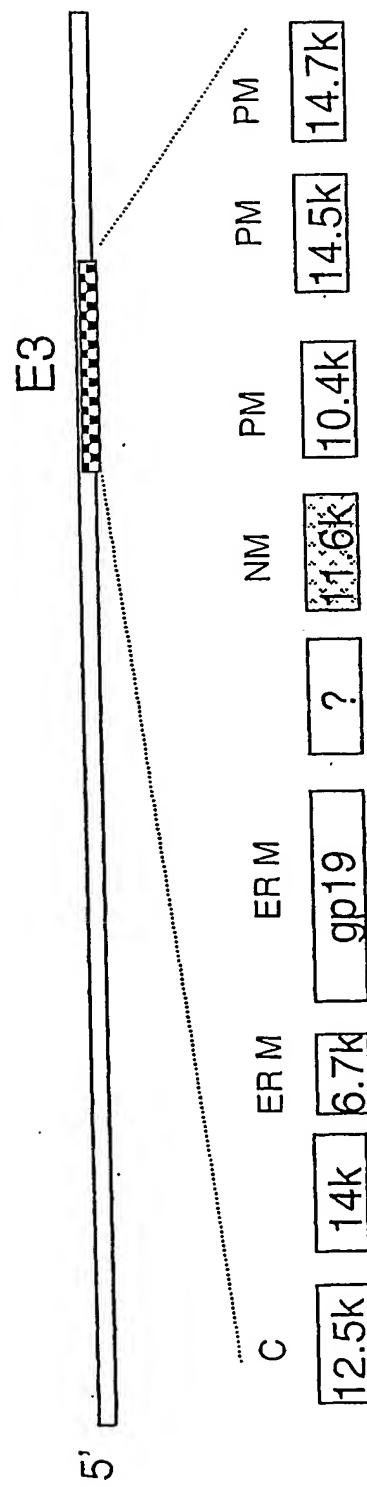


Figure 6

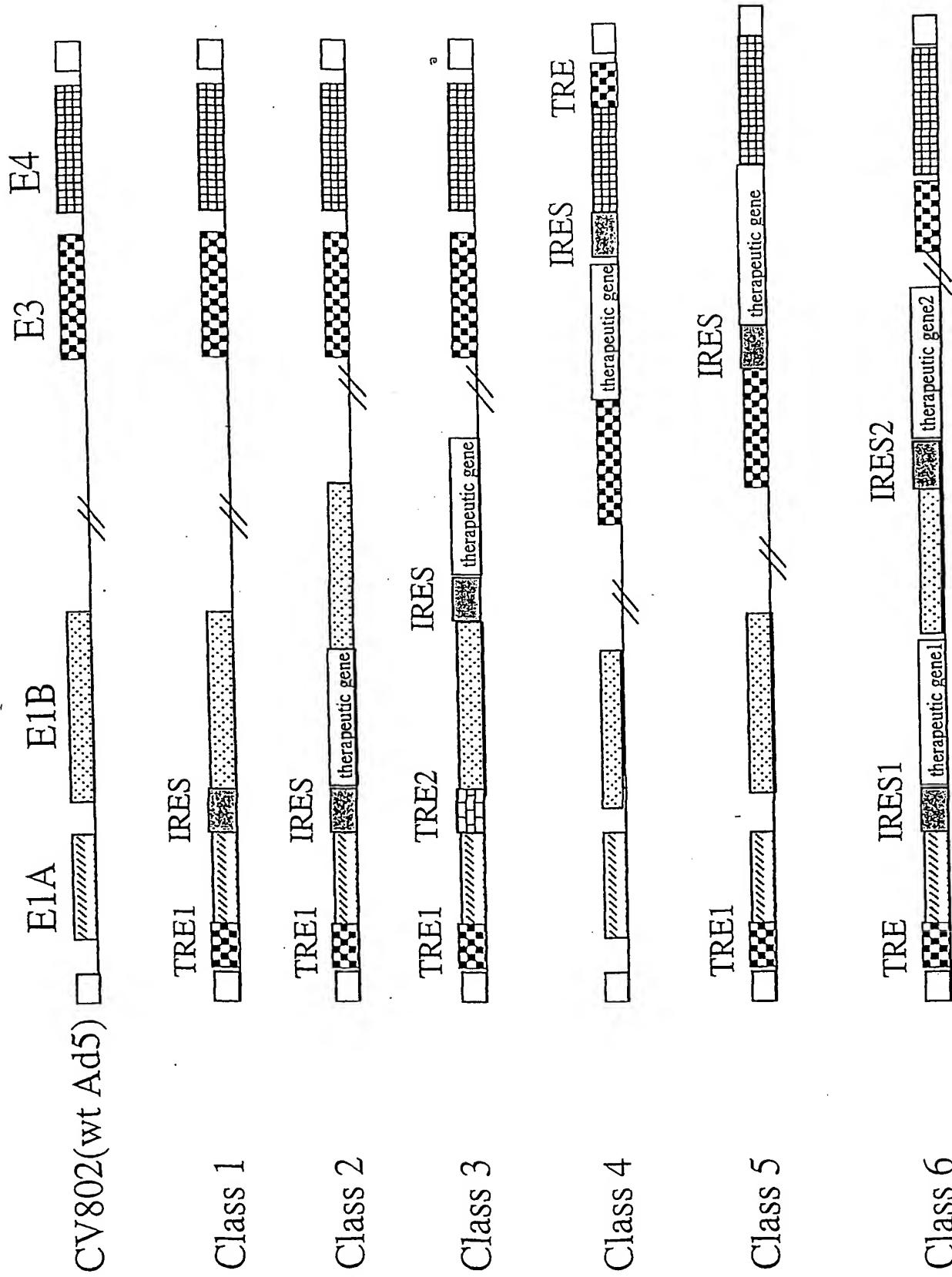
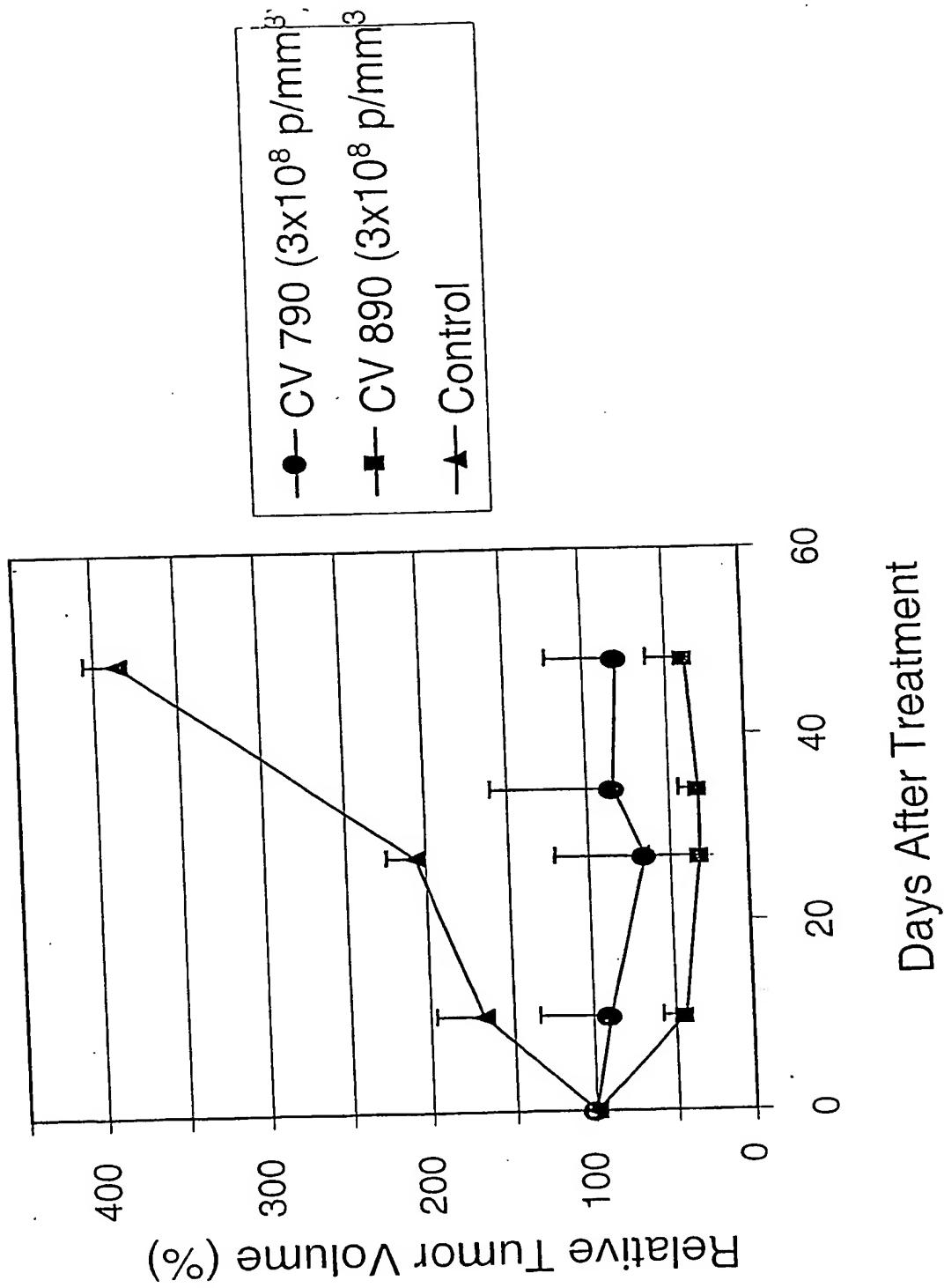


Figure 7

Figure 8



## FIG. 9

G ATG ACC GGC TCA ACC ATC GCG CCC ACA ACG GAC TAT CGC AAC ACC  
46 Met Thr Gly Ser Thr Ile Ala Pro Thr Thr Asp Tyr Arg Asn Thr  
1 5 10 15

ACT GCT ACC GGA CTA ACA TCT GCC CTA AAT TTA CCC CAA GTT CAT GCC  
94 Thr Ala Thr Gly Leu Thr Ser Ala Leu Asn Leu Pro Gln Val His Ala  
20 25 30

TTT GTC AAT GAC TGG GCG AGC TTG GAC ATG TGG TGG TTT TCC ATA GCG  
142 Phe Val Asn Asp Trp Ala Ser Leu Asp Met Trp Trp Phe Ser Ile Ala  
35 40 45

CTT ATG TTT GTT TGC CTT ATT ATT ATG TGG CTT ATT TGT TGC CTA AAG  
190 Leu Met Phe Val Cys Leu Ile Ile Met Trp Leu Ile Cys Cys Leu Lys  
50 55 60

CGC AGA CGC GCC AGA CCC CCC ATC TAT AGG CCT ATC ATT GTG CTC AAC  
238 Arg Arg Arg Ala Arg Pro Pro Ile Tyr Arg Pro Ile Ile Val Leu Asn  
65 70 75

CCA CAC AAT GAA AAA ATT CAT AGA TTG GAC GGT CTG AAA CCA TGT TCT  
286 Pro His Asn Glu Lys Ile His Arg Leu Asp Gly Leu Lys Pro Cys Ser  
80 85 90 95

CTT CTT TTA CAG TAT GAT TAA  
307 Leu Leu Leu Gln Tyr Asp  
100

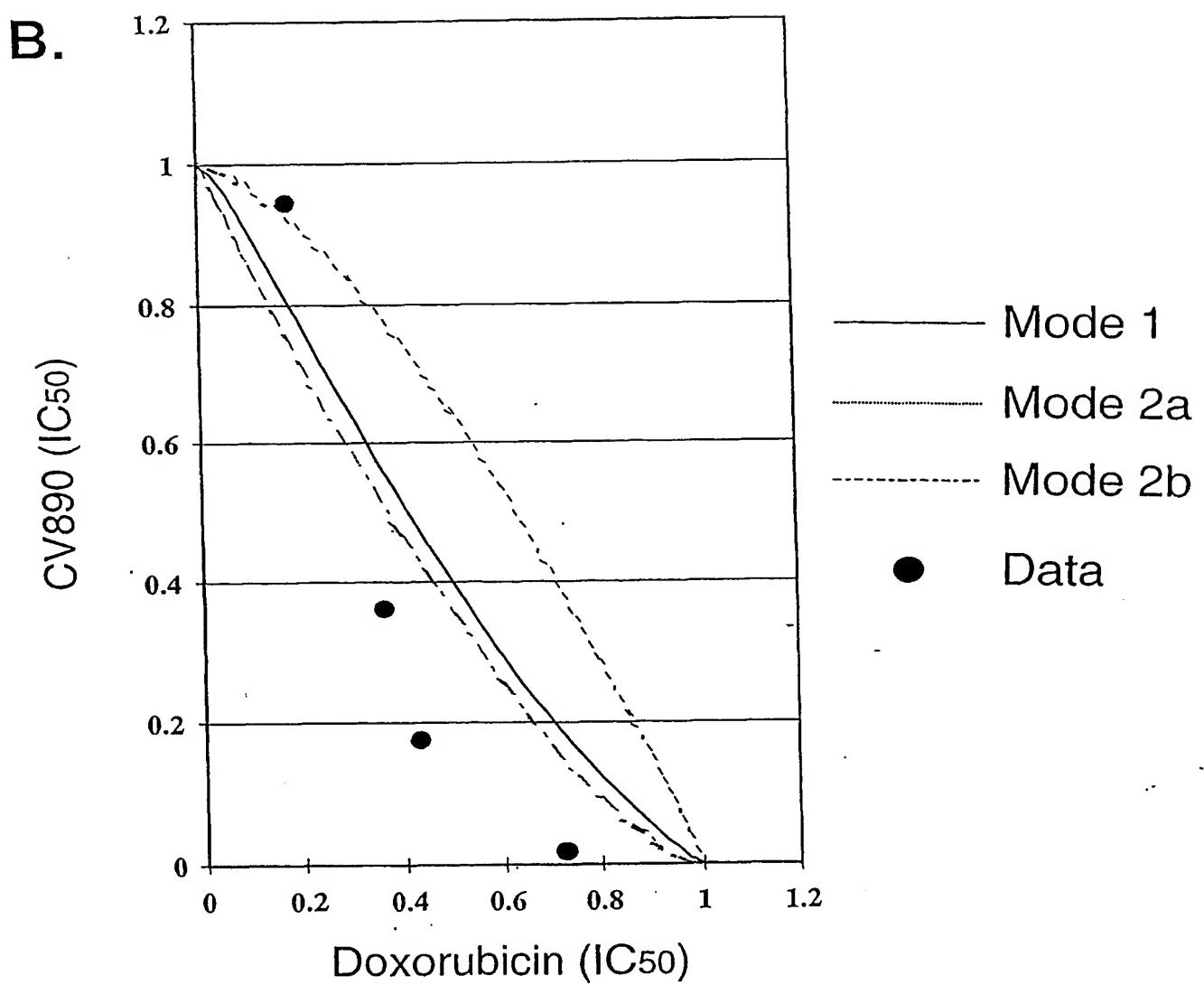


Figure 10

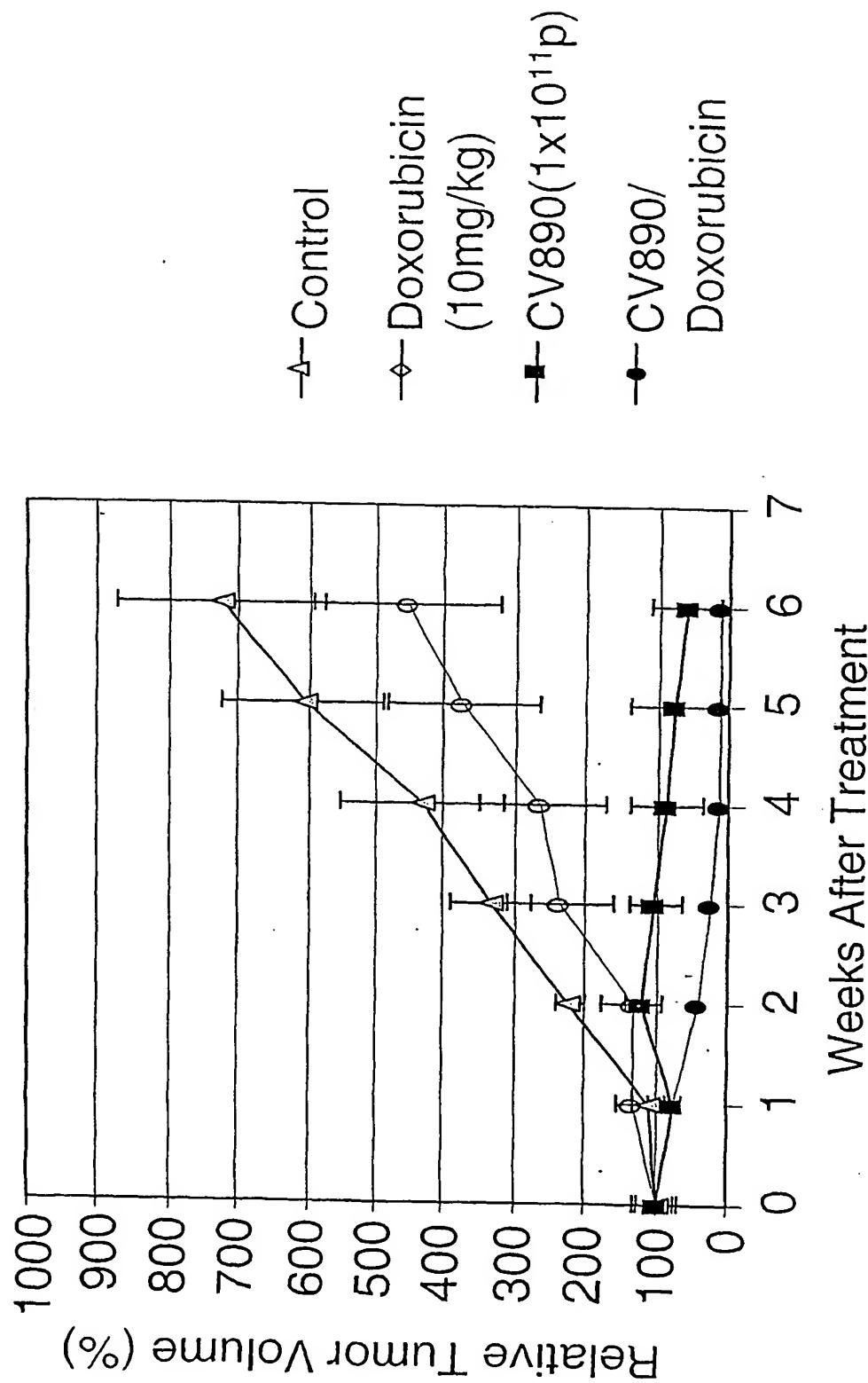
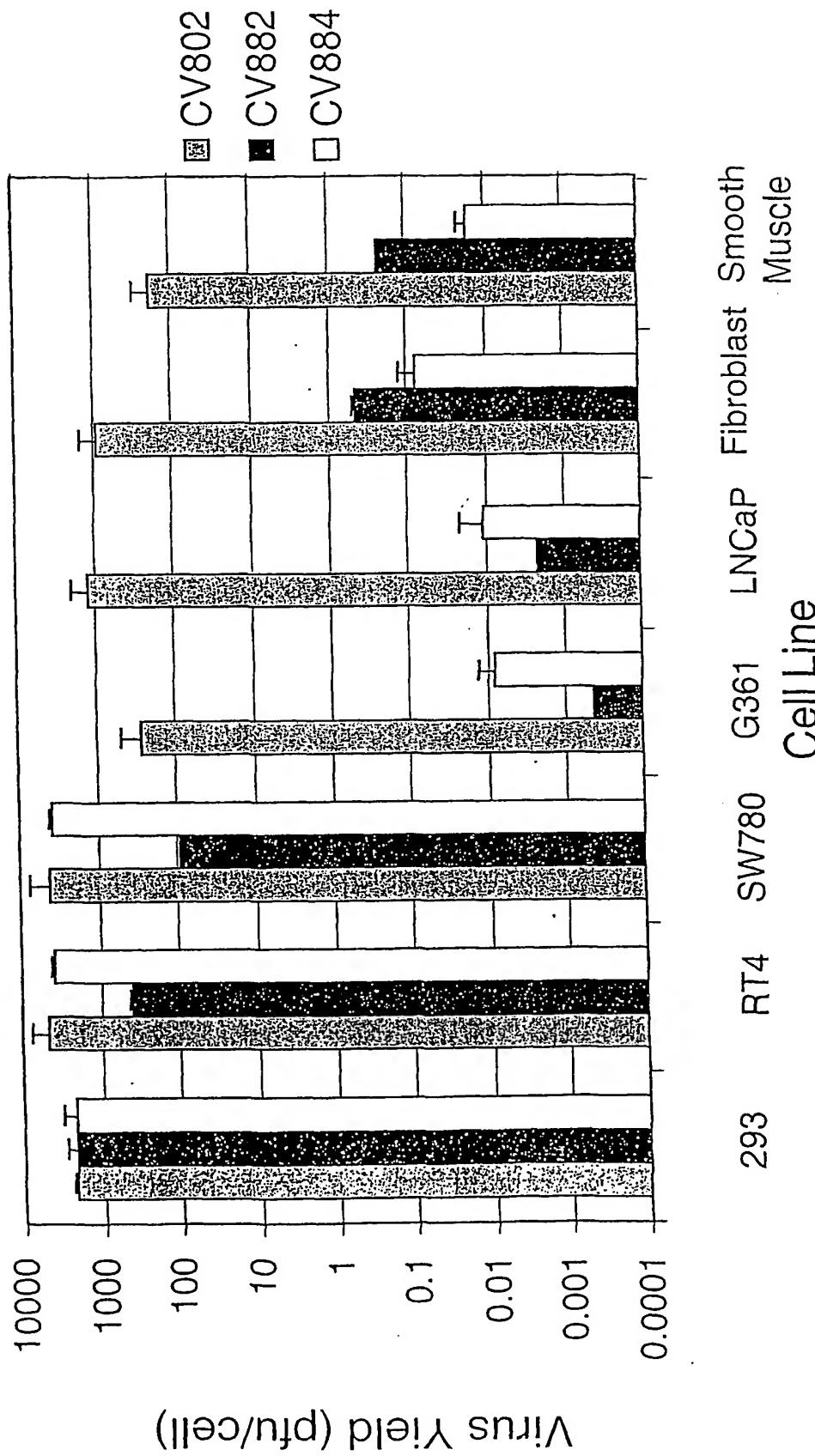


Figure 11

Figure 12  
Virus Yield of CV884



(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
4 October 2001 (04.10.2001)

PCT

(10) International Publication Number  
WO 01/073093 A3(51) International Patent Classification<sup>7</sup>: C12N 15/861, 15/63, 5/10, A61K 48/00, A61P 35/00

(74) Agent: JUDGE, Linda, R.; Cell Genesys, Inc., Intellectual Property Department, 342 Lakeside Drive, Foster City, CA 94404 (US).

(21) International Application Number: PCT/US01/09036

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(22) International Filing Date: 21 March 2001 (21.03.2001)

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/192,156 24 March 2000 (24.03.2000) US

(71) Applicant (for all designated States except US): CELL GENESYS, INC. [US/US]; 342 Lakeside Drive, Foster City, CA 94404 (US).

(72) Inventors; and

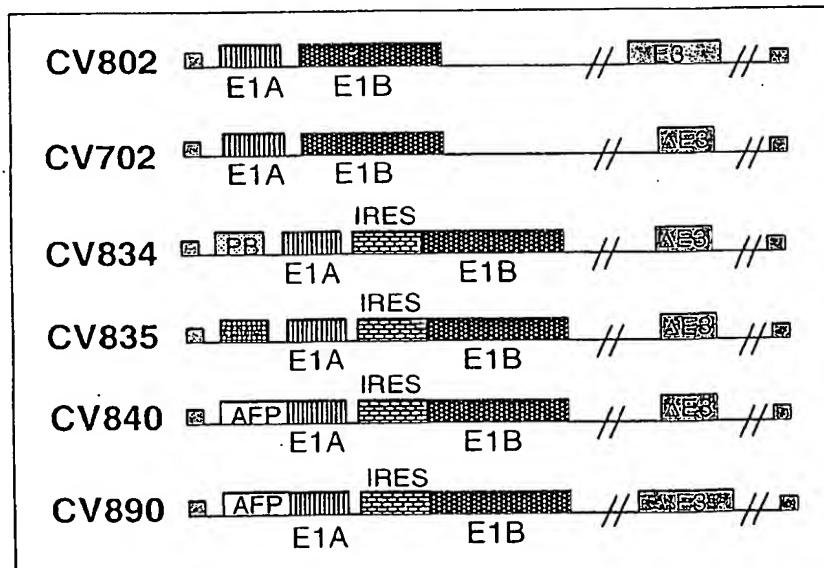
(75) Inventors/Applicants (for US only): YU, De-Chao [CN/US]; 352 Dolphin Isle, Foster City, CA 94404 (US). LI, Yuanhao [CA/US]; 568 Irvin Court, Palo Alto, CA 94306 (US). HENDERSON, Daniel, R. [US/US]; 955 Matadero Avenue, Palo Alto, CA 94306 (US).

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

[Continued on next page]

(54) Title: CELL-SPECIFIC ADENOVIRUS VECTORS COMPRISING AN INTERNAL RIBOSOME ENTRY SITE



WO 01/073093 A3

(57) Abstract: Disclosed herein are replication-competent adenovirus vectors comprising co-transcribed first and second genes under transcriptional control of a heterologous, target cell-specific transcriptional regulatory element (TRE), wherein the second gene is under translational control of an internal ribosome entry site. Methods for the preparation and use of such vectors are also provided. The vectors provide target cell-specific virus replication in applications such as cancer therapy and gene therapy.



(88) Date of publication of the international search report:  
6 September 2002

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

## INTERNATIONAL SEARCH REPORT

1st Application No

PCT/US 01/09036

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 7 C12N15/861 C12N15/63 C12N5/10 A61K48/00 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 7 C12N A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 06576 A (CALYDON INC ; SCHUUR ERIC R (US); YU DE CHAO (US); HERDENSON DANIEL) 11 February 1999 (1999-02-11) cited in the application claims 32,42 ---	1-32, 36-52, 55-58
A	WO 99 25860 A (NOVARTIS ERFIND VERWALT GMBH ; NOVARTIS AG (CH); HALLENBECK PAUL L) 27 May 1999 (1999-05-27) cited in the application ---	
A	WO 98 39464 A (LAMPARSKI HENRY G ; CALYDON INC (US); YU DE CHAO (US); HENDERSON DA) 11 September 1998 (1998-09-11) cited in the application --- -/-	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

28 March 2002

Date of mailing of the international search report

08.07.02

## Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax: (+31-70) 340-3016

Authorized officer

Lonnoy, O

## INTERNATIONAL SEARCH REPORT

Serial Application No

PCT/US 01/09036

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 46371 A (BRUCKHEIMER ELIZABETH M ;MCDONNELL TIMOTHY J (US); SWISHER STEPHEN) 16 September 1999 (1999-09-16) ---	
A	WO 98 37189 A (Q B I ENTERPRISES LTD ;YISSUM RES DEV CO (IL); ITIN AHUVA (IL); KE) 27 August 1998 (1998-08-27) cited in the application ---	
A	YU D-C ET AL: "The addition of adenovirus type 5 region E3 enables Calydon Virus 787 to eliminate distant prostate tumor xenografts" CANCER RES, vol. 59, no. 17, 1999, pages 4200-4203, XP002194692 ---	
T	LI Y ET AL: "A hepatocellular carcinoma-specific adenovirus variant, CV890, eliminates distant human liver tumors in combination with doxorubicin" CANCER RES, vol. 61, no. 17, 2001, pages 6428-6436, XP002194693 figure 1 -----	1-32, 36-52, 55-58

# INTERNATIONAL SEARCH REPORT

national application No.  
PCT/US 01/09036

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 56-58, as far as they relate to in vivo methods, are directed to methods of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-32, 36-52, 55-58 all totally

Remark on Protest

The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-32, 36-52, 55-58 (all totally)

A replication-competent adenovirus vector comprising first and second genes co-transcribed as a single mRNA wherein the first and the second genes are under transcriptional control of a heterologous, target cell-specific transcriptional regulatory element, wherein the second gene has a mutation in or deletion of its endogenous promoter and is under translational control of an internal ribosome entry site.

2. Claims: 33-35 (all totally)

An adenovirus vector comprising a gene under transcriptional control of a melanocyte-specific transcriptional regulatory element.

3. Claims: 53,54 (both totally)

An adenovirus vector comprising E1B under transcriptional control of a heterologous, target cell specific transcriptional control element, wherein E1B has a deletion of part or all of the 19-kDa region.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/09036

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9906576	A 11-02-1999	AU 744725 B2 AU 6345098 A AU 8692598 A EP 1007715 A2 EP 1002103 A1 JP 2002514074 T WO 9839464 A2 WO 9906576 A1		28-02-2002 22-09-1998 22-02-1999 14-06-2000 24-05-2000 14-05-2002 11-09-1998 11-02-1999
WO 9925860	A 27-05-1999	AU 1755299 A CA 2310563 A1 WO 9925860 A1 EP 1032696 A1 JP 2002507382 T		07-06-1999 27-05-1999 27-05-1999 06-09-2000 12-03-2002
WO 9839464	A 11-09-1998	AU 745847 B2 AU 6187698 A AU 744725 B2 AU 6345098 A EP 1017836 A2 EP 1007715 A2 JP 2002514074 T JP 2001515351 T US 6197293 B1 WO 9839464 A2 WO 9839466 A2 AU 745600 B2 AU 6445698 A EP 0968298 A2 JP 2002514075 T WO 9839467 A2 AU 8692598 A EP 1002103 A1 WO 9906576 A1		11-04-2002 22-09-1998 28-02-2002 22-09-1998 12-07-2000 14-06-2000 14-05-2002 18-09-2001 06-03-2001 11-09-1998 11-09-1998 21-03-2002 22-09-1998 05-01-2000 14-05-2002 11-09-1998 22-02-1999 24-05-2000 11-02-1999
WO 9946371	A 16-09-1999	AU 3183799 A CA 2322663 A1 EP 1070122 A2 WO 9946371 A2		27-09-1999 16-09-1999 24-01-2001 16-09-1999
WO 9837189	A 27-08-1998	AU 6667798 A EP 1012268 A1 JP 2002513285 T WO 9837189 A1		09-09-1998 28-06-2000 08-05-2002 27-08-1998

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**